Optimization of Potassium Hydroxide Combined Urea Pretreatment and Enzymatic Hydrolysis of Wheat Straw Using Response Surface Methodology for Improving Sugar Production

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To improve sugar yields from wheat straw (WS), response surface methodology (RSM) was adopted to optimize potassium hydroxide combined urea pretreatment and enzymatic hydrolysis of WS. Glucose and xylose yields from the pretreated WS were used as responses during the whole optimization. Potassium hydroxide concentration, time and temperature during pretreatment were found to have significant effects on sugar yields. Sugar yields could be enhanced while WS was pretreated using 45 g/L potassium hydroxide solution containing 15 g/L urea with solid to liquid ratio of 1:5 (g/mL) at 74.0 °C for 50 min. Cellulose recovery, hemicellulose recovery, and lignin removal after optimization were 98.1%, 72.6%, and 75.8%, respectively. In addition, enzyme loading, biomass loading, and reaction time during enzymatic hydrolysis also had significant effects on sugar yields. Maximal yields of glucose (610.25 mg/gds, miligram per gram dry substrate) and xylose (221.26 mg/gds) could be achieved while hydrolysis was carried out at 50 °C for 32.8 h with 141 g/L of biomass loading, 8.1 FPU/gds (filter paper activity unit per gram dry substrate) of enzyme loading and 0.4% (w/v) of polysorbate 80. The corresponding cellulose conversion and hemicellulose conversion were 97.2% and 90.4%, respectively.

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Keywords: Wheat straw; Optimization; Potassium hydroxide combined urea pretreatment; Enzymatic hydrolysis; Response surface methodology

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INTRODUCTION

The increase of global energy demand and the escalating problems of environmental pollution caused by traditional energy consumption has promoted the development and utilization of renewable fuels (Shao *et al.* 2023). Conversion of abundant lignocellulosic biomass to bioethanol presents a promising option for improving energy security along with reducing the emission of green-house gases, preventing the consequent increase in the average global temperature, and producing a sustainable energy (Dong *et al.* 2019). Lignocellulosic biomass is mainly composed of cellulose, hemicellulose, and lignin. Among them, cellulose and hemicellulose are mainly responsible for glucose and xylose production, which can be utilized to produce bioethanol by yeast strains. However, the complex and dense structure formed by these components makes it difficult to be

directly utilized by hydrolases (Zhang *et al.* 2020b). Therefore, pretreatment to break the strong barrier such as natural recalcitrance, the presence of lignin, and the high crystallinity of cellulose of lignocelluloses is necessary before enzymatic hydrolysis (Kang *et al.* 2018; Sewsynker-Sukai *et al.* 2020). Multiple pretreatments have been explored including physical (microwave, steam explosion, milling), chemical (oxidative, acidic, alkaline), or biological (fungal, enzymatic) methods (Shao *et al.* 2023). Among these methods, alkali pretreatment, especially sodium hydroxide method, is regarded as an economical and effective technology due to its high delignification, low cost, and effective increasing of the accessibility of cellulose (Wang *et al.* 2019). However, the disadvantage of sodium hydroxide pretreatment is production of vast amounts of black liquor. The presence of a large amount of Na+ in black liquor will aggravate soil salinization and water pollution, which restrains sodium hydroxide pretreatment industrial application (Wang *et al.* 2022). In order to reduce environmental hazards and develop a sustainable process, researchers have proposed the use of potassium hydroxide instead of sodium hydroxide as the alkaline reagent, because the black liquor containing potassium discharged after treatment can be used as liquid fertilizer for plant growth and soil amendment (Liu *et al.* 2015; Jaffar *et al.* 2016; Ji *et al.* 2017). It is also reported that employing potassium hydroxide for pretreatment is more sustainable and economical than sodium hydroxide, considering the high costs associated with chemical recovery (Chi *et al.* 2019). Therefore, potassium hydroxide pretreatment is a relatively more promising pretreatment method, which was preferred compared with sodium hydroxide pretreatment.

Urea is an essential nutrient for plant growth by containing nitrogen and it can be transported, stored, and used without special equipment or conditions. It is reported that urea pretreatment is favorable for the enzymatic hydrolysis of corn stover (Wang *et al.* 2018). Whereas numerous studies indicate that it is difficult for urea pretreatment alone to achieve the expected degradation effect, and the combination of urea and other pretreatments is more effective than urea pretreatment alone (Zahoor *et al.* 2021a; Wu *et al.* 2023). Some studies mentioned that urea can be used as an additive for sodium hydroxide pretreatment of substrates such as bamboo (Li *et al.* 2010), rice straw (Dong *et al.* 2018), and softwood spruce and hardwood birch (Mohsenzadeh *et al.* 2012). In fact, urea can also be combined with potassium hydroxide and this method has been adopted to pretreat lignocellulosic substrates such as sugarcane bagasse (Wang *et al.* 2022), corn stover (Zahoor *et al.* 2021a), and rice straw (Zahoor *et al.* 2021b). The produced wash liquid can also be used as fertilization in crop production (Song *et al.* 2020; Zahoor *et al.* 2021a;) and the liquid fertilizer can be absorbed more easier and faster, which can promote crop growth more effectively (Ji *et al.* 2017). According to the report described by Zahoor *et al.* (2021a), the liquid waste generated from potassium hydroxide combined urea pretreatment of corn stover with proper dilution and showed significant enhancement in the length and biomass weight of wheat, maize, tomato, chili, and tobacco. In addition, potassium hydroxide pretreatment with addition of urea can result in higher delignification and higher cellulose recovery compared with sole potassium hydroxide pretreatment and sole urea pretreatment (Zahoor *et al.* 2021a). During combined pretreatment, urea can also effectively cleave the chemical linkage between lignin and polysaccharides, and partially remove lignin, facilitating delignification by potassium hydroxide (Yoo *et al.* 2016). Even so, holocellulose including cellulose and hemicellulose can also be degraded during delignification. Therefore, it is necessary to optimize pretreatment conditions to realize high levels of retention of holocellulose and delignification simultaneously.

After pretreatment, cellulose can be hydrolyzed by synergistic actions of β-1, 4-endoglucanases (EC 3.2.1.4), β-1, 4-exoglucanases (EC 3.2.1.91), and β-D-glucosidases (EC 3.2.1.21) (Steffien *et al.* 2014). Hemicellulose can also be hydrolyzed by coordination of endoxylanases (EC 3.2.1.8) and β-xylosidases (EC 3.2.1.37) (Garai and Kuma 2013). The produced sugar can efficiently be used for ethanol fermentation either by separate hydrolysis and fermentation (SHF) or simultaneous saccharification and fermentation (SSF). However, the optimal temperature for the yeast differs from that of cellulolytic and hemicellulolytic enzymes, which means that the conditions used in SSF cannot be optimal for both the enzymes and the yeast. The differences might result in lower efficiency and lower product yield. Hence, for better efficiency of ethanol production, the approach of SHF is preferred (Karin *et al.* 2007; Saini *et al.* 2013). It is obvious that enzymatic hydrolysis to produce fermentable sugar is one of the most important steps in bioethanol production (Steffien *et al.* 2014). Whereas, enzymes activities can be influenced by temperature, pH, biomass loading, enzyme loading, addition of surfactant and so on. Hence it is necessary to optimize enzymatic hydrolysis to enhance sugar yields, hydrolysis efficiency, and reduce input cost.

Response surface methodology (RSM) is an effective optimization tool wherein many factors and their interactions affecting the response can be identified with fewer experimental trials (Hu *et al.* 2018). In North China, wheat straw (WS) is a common and abundant lignocellulosic substrate, which make it possible to be applied in bioethanol production in large scale. To improve sugar yields from WS, potassium hydroxide combined urea pretreatment and enzymatic of WS were optimized using RSM in this work.

EXPERIMENTAL

Biomass and Chemicals

Wheat straw (WS) was obtained from a nearby farm in Liaocheng, Shandong Province, China. WS was cut to 1-2 cm length without grinding and was dried at 85 °C until constant weight. Potassium hydroxide (AR), Urea (AR), D-glucose (AR), acetic acid (AR), sodium acetate (AR), and Polysorbate 80 (CP) were purchased from Xilong Scientific Co., Ltd., Shantou, China. D-xylose (BR) was purchased from Sinopharm Chemical Reagent Co., Ltd., Shanghai, China.

Enzymes Production, Extraction, and Activities Assays

The crude cellulases preparation containing 6.13 filter paper activity units/mL (6.13 FPU/mL) and 889.18 xylanase activity unit/mL (889.18 XU/mL) was produced by *Aspergillus niger* HQ-1 (accession number of ITS sequence: HQ891869) according to the methods in our previous report (Zhang *et al.* 2012; Zhang and Wu 2023).

Optimization of Potassium Hydroxide Combined Urea Pretreatment of Wheat Straw

Pretreatment was carried out in 250 mL Erlenmeyer flasks by mixing WS with 100 mL potassium hydroxide-urea solution. At first, various pretreatment conditions were designed using Plackett-Burman design (PBD). Variables including potassium hydroxide concentration (8, 15, g/L), urea concentration (8, 15, g/L), pretreatment temperature (40 °C, 50 °C), pretreatment time (20, 30, min) and solid to liquid ratio (1:5, 1:4, g/mL) at two

levels of variation (-1: low; +1: high) were chosen in PBD (Table S1). The obtained WS after pretreatment under different conditions were washed and filtered with double-distilled water until neutrality. After being dried in an oven at 80 °C until constant weight, the pretreated WS was hydrolyzed by cellulases preparation under initial enzymatic hydrolysis conditions and the yields of glucose and xylose were adopted as responses. Then, according to the analysis results of PBD, the steepest ascent method was used to investigate the optimal regions of the significant variables including potassium hydroxide concentration, pretreatment temperature and time (Table S2). Finally, the optimal values of the significant variables were investigated using central composite design (CCD). Five levels including -1.682, -1, 0, +1 and +1.682 (coded values) of the three variables including potassium hydroxide concentration (19.7, 30, 45, 60, 70.2, g/L), temperature (58, 65, 75, 85, 92, °C) and time (33, 40, 50, 60, 67, min) during pretreatment were adopted (Table S3). 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 Eq. 1,

$$Y = \beta 0 + \sum \beta ixi + \sum \beta iixi2 + \sum \beta ijxixj \quad (1)$$

where Y was the predicted response, x1, x2, and x3 the coded variables, β0 the intercept term, βi linear coefficient, βii the squared coefficient, and βij the interaction coefficient.

Chemical Compositions Analyses and Calculations

Cellulose, hemicellulose, and lignin in raw WS and the obtained WS pretreated under the optimal conditions were determined according to the methods described by Aravantinos-Zafiris *et al.* (1994). Solid recovery (SR), cellulose recovery (CR), hemicellulose recovery (HCR), and lignin removal (LR) were calculated as follows,

$$SR\ (\%) = (W1 / W0) \times 100\% \quad (2)$$
$$CR\ (\%) = (C1 \times SR / C0) \times 100\% \quad (3)$$
$$HCR\ (\%) = (HC1 \times SR / HC0) \times 100\% \quad (4)$$
$$LR\ (\%) = 1- [(L1 \times SR) / L0] \times 100\% \quad (5)$$

where W1 is the dry weight of WS recovered after pretreatment (g), W0 is the dry weight of WS before pretreatment (g), C0 and C1 are the amounts of cellulose in raw and pretreated WS expressed in g/g, respectively; HC0 and HC1 are the amount of hemicellulose in raw and the pretreated WS expressed in (g/g), respectively; L0 and L1 are the amount of lignin in raw and the pretreated WS expressed in (g/g), respectively.

Initial Enzymatic Hydrolysis Conditions and Optimization of Enzymatic Hydrolysis

Initial enzymatic hydrolysis conditions

Enzymatic hydrolysis was performed at 50 °C, 100 rpm for 12.0 h in 250 mL Erlenmeyer flasks containing 100 mL of sodium acetate buffer (50 mM, pH 4.8), 0.4% (w/v) polysorbate 80 and 80 g/L pretreated WS. Antibiotics tetracycline (40 μg/mL) and cycloheximide (30 μg/mL) were supplemented to prevent microbial contamination. Cellulases preparation was added at 2.0 FPU/gds (filter paper activity unit per gram dry substrate). After completing hydrolysis, residues were separated by centrifugation at 10000 rpm for 10 min and the obtained supernatant was used to determine the released glucose and xylose yields.

The monosaccharides (glucose and xylose) were determined *via* HPLC as described previously (Zhang and Wu 2023). The yields of glucose and xylose were expressed as milligrams per gram dry substrate (mg/gds).

Optimization of Enzymatic Hydrolysis of the Pretreated Wheat Straw

The obtained WS pretreated under the optimal conditions was used as substrate during optimization of enzymatic hydrolysis. At the first step, different enzymatic hydrolysis conditions were also designed using PBD. High level (+1) and low level (-1) of six variables including enzyme loading (2.0, 4.0, FPU/gds), biomass loading (70, 95, g/L), polysorbate 80 concentration (0.2%, 0.4%, w/v), reaction temperature (50, 55, °C), reaction pH (4.4, 4.8) and reaction time (12.0, 18.0, h) were adopted in PBD (Table S4). Then, based on analysis results of PBD, the optimal regions of the screened significant variables including enzyme loading, biomass loading, and reaction time were investigated using the steepest ascent method (Table S5). Finally, the optimal values of the significant variables were investigated using Box-Behnken design (BBD). Three levels denoted as high (+1), middle (0) and low level (-1) of the variables including enzyme loading (6.0, 8.0, 10.0, FPU/gds), biomass loading (120, 145, 170, g/L) and reaction time (28.0, 33.0, 38.0, h) were adopted (Table S6). 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. (6):

$$Y = \beta0 + \beta1X1 + \beta2X2 + \beta3X3 + \beta11X12 + \beta22X22 + \beta33X32 + \beta12X1X2 + \beta13X1X3 + \beta23X2X3 \quad (6)$$

in which, Y was the predicted response, X1, X2, and X3 the coded variables, β0 intercept, β1, β2, and β3 the linear coefficients, β11, β22, and β33 the squared coefficients and β12, β13, and β23 the interaction coefficients.

Calculations for Enzymatic Hydrolysis

Cellulose conversion and hemicellulose conversion were calculated as follows,

$$\text{Cellulose conversion (\%)} = (Mg \times 0.9 / Mc) \times 100\% \quad (7)$$

$$\text{Hemicellulose conversion (\%)} = (Mx \times 0.88 / Mh) \times 100\% \quad (8)$$

where, Mg is the amount of glucose from the pretreated WS (mg/gds); Mc is the amount of cellulose in the pretreated WS (mg/gds); Mx is the amount of xylose from the pretreated WS (mg/gds); Mh is the amount of hemicellulose in the pretreated WS (mg/gds); 0.9 and 0.88 are the conversion factors of glucose and xylose, respectively.

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. The significance was tested at various probability levels using the F test ($P \leq 0.05$).

RESULTS AND DISCUSSION

Optimization of Potassium Hydroxide Combined Urea Pretreatment of Wheat Straw

An ideal pretreatment should remove more lignin and maximize the retention of holocellulose. Degradation of holocellulose occurs during delignification, which indicates that lignin reduction and recovery of cellulose, and hemicellulose cannot be adopted as responses for pretreatment optimization. Therefore, in this work, yields of glucose and xylose from the pretreated WS were used as responses for pretreatment optimization, as the main aim of optimization is enhancing enzymatic digestibility of holocellulose in WS. As shown in Table 1, variables including potassium hydroxide concentration, temperature, and time during pretreatment had significant and positive effects on sugar yields. Increase of potassium hydroxide, temperature, and time can enhance delignification, which was a benefit to sugar yields. Zahoor *et al.* (2021b) also mentioned that variables including potassium/urea concentration, temperature, time, and solid to liquid ratio during pretreatment had significant effects on sugar yields from rice straw.

Temperature and time during pretreatment also had significant effects on sugar yields from sugarcane bagasse (Wang *et al.* 2019; Zhang *et al.* 2022) and poplar (*Populus deltoides*) biomass (Rawat *et al.* 2013). According to the research described by Sharma *et al.* (2013), potassium hydroxide concentration and temperature during pretreatment also had a positive effect on delignification of switchgrass, which could enhance sugar yields. Whereas potassium hydroxide concentration and temperature during pretreatment had insignificant effect on sugar yields from *Parthenium hysterophorus* (Kumar *et al.* 2023), and time during pretreatment had an insignificant effect on sugar yields from cocoa (*Theobroma cacao* L) pod husks (Ouattara *et al.* 2023). In addition, temperature and time during pretreatment had insignificant effects on sugar yields from jute (*Corchorus olitorius* L.) biomass (Sharma *et al.* 2023). The difference of significant variables during pretreatment on sugar yields was perhaps related to the adopted pretreatment methods and types of lignocellulosic substrates. Among the insignificant variables, urea concentration had positive effect on sugar yields as relatively higher urea concentration can also promote delignification. In addition, the insignificant effect of urea concentration on sugar yields indicated that potassium hydroxide played a main role in delignification during combined pretreatment in this work. Solid to liquid ratio during pretreatment had insignificant effect on sugar yields in this work. Similar results existed in the pretreatment of sugarcane bagasse described by Zhang *et al.* (2022). In addition, solid to liquid ratio during pretreatment has a negative effect on sugar yield in this work, as the lowering of the ratio can lead to relatively higher potassium hydroxide and urea concentration, which could intensify delignification. Similar results existed in the pretreatment of sugarcane bagasse described by Xu *et al.* (2015). Therefore, solid to liquid ratio (1:5, g/mL) and 15 g/L urea were adopted in the following steps.

Table 1. Coefficients of Regression for Glucose and Xylose Yields during Optimization of Pretreatment

Terms	Y_1 (Glucose)	Y_2 (Xylose)
Constant	129.056	25.0483
A (Potassium hydroxide concentration)	9.609**	3.0417**
B (Urea concentration)	0.589	0.0717
C (Pretreatment temperature)	7.494**	3.6233**
D (Pretreatment time)	7.216**	2.7033**
E (Solid to liquid ratio)	-1.061	-0.2250
Lack of fit	0.114	0.110
R^2	97.38%	99.29%
Adj-R^2	95.42%	98.75%

Outline criterion: 0.05; ** Significant at 1% level

Results of the steepest ascent method (Table S2) indicated that glucose and xylose yields reached the plateau while potassium hydroxide concentration, temperature, and time during pretreatment were 45 g/L, 75.0 °C and 50.0 min, respectively. Statistical analysis of CCD in Table 2 showed that linear terms including x1, x2, and x3 and square terms including x12, x22, and x32 had significant effects on glucose and xylose yields. Whereas, interaction terms including x1x2, x1x3, and x2x3 had insignificant effects on glucose and xylose yields. P-values of the models (P = 0.000; P = 0.000) and lack of fit (P = 0.104; P = 0.126) along with high F-values of the models (F = 270.87; F = 241.10) and lack of fit (F = 3.37; F = 3.01) indicated that glucose and xylose yields could be predicted by the models, respectively. In addition, high values of R2 (99.6%; 99.5%) and the adjusted R2 (99.2%; 99.1%) also indicated the accuracy of the models for predicting glucose and xylose yields, respectively.

Table 2. Estimated Regression Coefficients for Glucose and Xylose Yields during Optimization of Pretreatment

Terms	Y_1 (Glucose)	Y_2 (Xylose)
Constant	279.312	77.5122
x_1 (Potassium hydroxide concentration)	6.302**	-2.3674**
x_2 (Pretreatment temperature)	-3.319**	-1.5806**
x_3 (Pretreatment time)	6.239**	-2.0841**
$x_1 \times x_1$ (Potassium hydroxide concentration × potassium hydroxide concentration)	-23.660**	-8.3068**
$x_2 \times x_2$ (Pretreatment temperature × pretreatment temperature)	-19.362**	-6.7918**
$x_3 \times x_3$ (Pretreatment time × pretreatment time)	-15.874**	-9.2543**
$x_1 \times x_2$ (Potassium hydroxide concentration × pretreatment temperature)	-0.100	0.4838
$x_1 \times x_3$ (Potassium hydroxide concentration × pretreatment time)	1.317	-0.1688

$x_2 \times x_3$ (Pretreatment temperature × pretreatment time)	0.175	-0.1562
R^2	99.6%	99.5%
Adj-R^2	99.2%	99.1%
Lack of fit	0.104	0.126

Outline criterion: 0.05; ** Significant at 1% level

Response surface plots and corresponding counter plots of the combined effects of each independent variable's pair on glucose and xylose yields were shown in Fig. 1 and Fig. 2, respectively. Figure 1 (a1, b1) and Fig. 2 (a1, b1) indicated that the optimal regions of potassium hydroxide concentration (x1) and temperature (x2) for glucose and xylose yields were 40 g/L to 48 g/L and 72 °C to 78 °C, respectively, while time (x3) was fixed at its middle level (50 min). Figure 1 (a2, b2) and Fig. 2 (a2, b2) indicated that the optimal regions of potassium hydroxide concentration (x1) and time (x3) for glucose and xylose yields were 40 g/L to 48 g/L and 48 min to 54 min, respectively, while temperature (x2) was fixed at its middle level (75.0 °C). Figure 1 (a3, b3) and Fig. 2 (a3, b3) indicated that the optimal regions of temperature (x2) and time (x3) for glucose and xylose yields were 72 °C to 78 °C and 48 min to 54 min, respectively, while potassium hydroxide concentration (x1) was fixed at its middle level (45 g/L). According to canonical analysis, maximal glucose yield (280.52 mg/gds) could be obtained after pretreatment using 47.1 g/L potassium hydroxide solution (x1 = 0.13898) at 74.1 °C (x2 = -0.08516) for 52 min (x3 = 0.20180). Maximal xylose yield (77.89 mg/gds) could be obtained after pretreatment using 42.8 g/L potassium hydroxide solution (x1 = -0.14488) at 73.8 °C (x2 = -0.12025) for 48.9 min (x3 = -0.11026).

The corresponding regression models for glucose and xylose yields during optimization of pretreatment conditions are given in Eq. 9 and Eq. 10,

$$Y1 = 279.312 + 6.302x1 - 3.319x2 + 6.239x3 - 23.660x12 - 19.362x22 - 15.874x32 - 0.100x1x2 + 1.317x1x3 + 0.175x2x3 \quad (9)$$

$$Y2 = 77.5122 - 2.3674x1 - 1.5806x2 - 2.0841x3 - 8.3068x12 - 6.7918x22 - 9.2543x32 + 0.4838x1x2 - 0.1688x1x3 - 0.1562x2x3 \quad (10)$$

whereas, Y1 and Y2 were predicted glucose and xylose yields, and x1, x2, and x3 were codes of potassium hydroxide concentration, pretreatment temperature, and time, respectively.

To validate the predicted conditions, after adjustment, WS was pretreated using 45 g/L potassium hydroxide solution at 74.0 °C for 50 min. Yields of glucose (281.41 mg/gds) and xylose (78.53 mg/gds) (average of three replicates) were obtained, which were in close proximity with the predicted glucose (280.52 mg/gds) and xylose (77.89 mg/gds) yields, respectively.

In addition, contents of cellulose, hemicellulose, and lignin in raw WS (dry weight 20.00 g) were 43.52%, 22.41%, and 9.80%, respectively. After pretreatment, contents of cellulose, hemicellulose, and lignin in the pretreated WS (dry weight 15.10 g) were 56.52%, 21.55%, and 3.14%, respectively. After calculation, pretreatment resulted in 75.5% of solid recovery, 98.1% of cellulose recovery, 72.6% of hemicellulose recovery, and 75.8% of lignin removal, respectively.

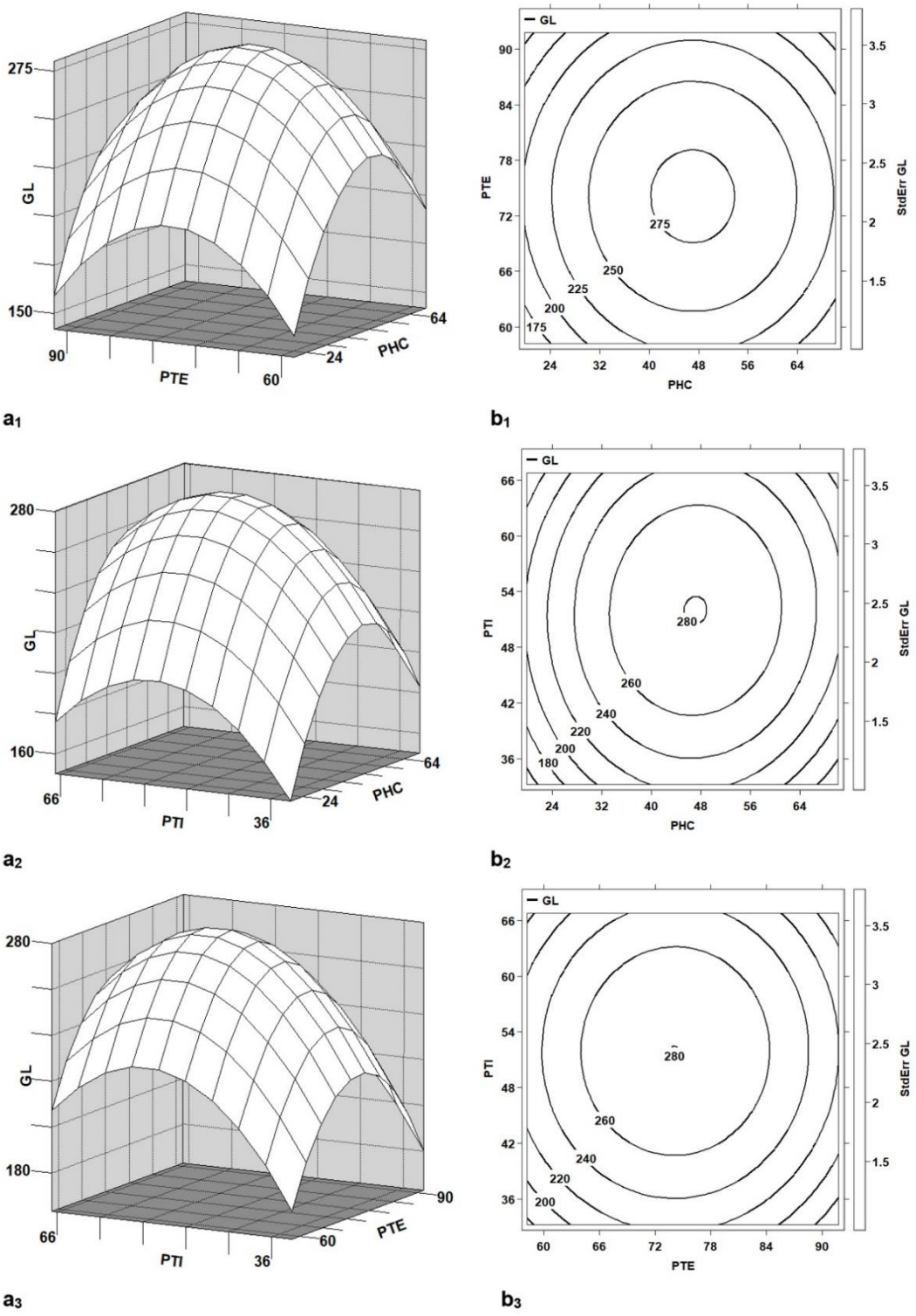

Fig. 1. Response surface plots (a) and contour plots (b) of interaction effect of each independent variable's pair on glucose (Y1). (a1, b1): PHC (Potassium hydroxide concentration) and PTE (pretreatment temperature); (a2, b2): PHC (Potassium hydroxide concentration) and PTI (pretreatment time); (a3, b3): PTE (pretreatment temperature) and PTI (pretreatment time)

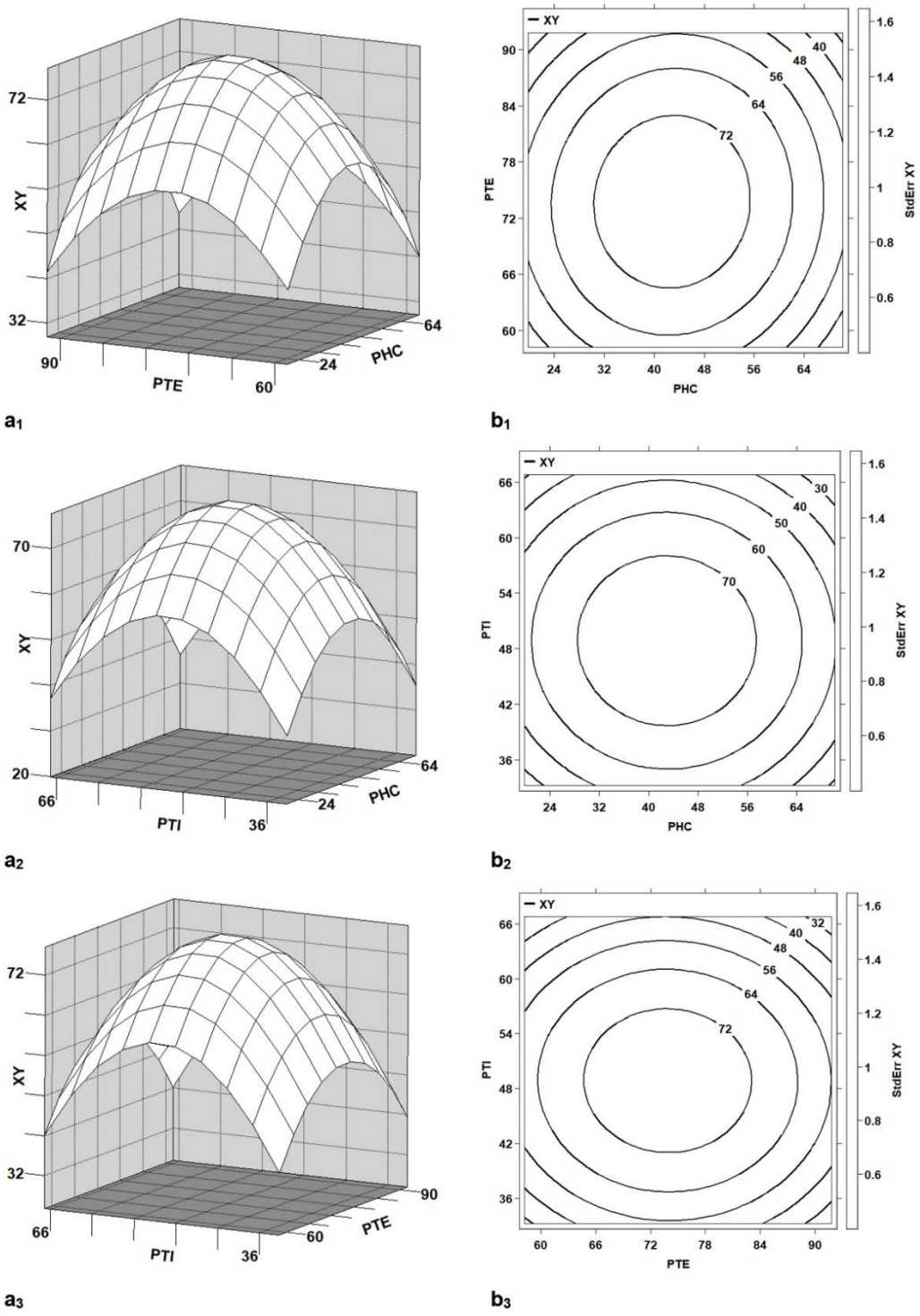

Fig. 2. Response surface plots (a) and contour plots (b) of interaction effect of each independent variable's pair on xylose (Y2). (a1, b1): PHC (Potassium hydroxide concentration) and PTE (pretreatment temperature); (a2, b2): PHC (Potassium hydroxide concentration) and PTI (pretreatment time); (a3, b3): PTE (pretreatment temperature) and PTI (pretreatment time)

As shown in Table 3, lignin removal including 87.6% (Wu *et al.* 2023) and 86.9% (Xie *et al.* 2020) were higher than that (75.8%) in this work, however, lower cellulose recovery (76.6%, 80.2%) and hemicellulose recovery (27.4%, 36.7%) existed in those two reports, which indicated that more holocellulose were degraded during pretreatment. Lignin removal (75.8%) in this work were relatively more competitive than 53.5% (Wang *et al.* 2022), 55.6% (Zahoor *et al.* 2021a), 51.4% (Zahoor *et al.* 2021b), 62.7% (Jiang *et al.* 2018), 65.5% (Qi *et al.* 2019a), 46.0% (Qi *et al.* 2019b) and 35.6% (Tang *et al.* 2020) in the other seven reports. In addition, cellulose recovery (98.1%) and hemicellulose recovery (72.6%) in this work were more competitive than those in the other nine reports, which indicated that the pretreated WS could provide a prerequisite for maximize sugar yields from WS in the subsequent enzymatic hydrolysis.

Comparisons of pretreatment conditions among different reports are shown in Table 3. Direct comparison of temperature and time during pretreatment among different reports was not available, as higher temperature always corresponded with shorter time, and lower temperature corresponded with longer time, respectively. Among various pretreatment conditions for WS in Table 3, temperature (80 °C) and time (30 min) during pretreatment of wheat straw described by Jiang *et al.* (2018) were relatively more competitive than those (74.0 °C, 50 min) in this work, adoption of higher sodium hydroxide concentration (80 g/L) and lower solid to liquid ratio (1:10, g/mL) in that work will enhance input costs of reagent and reaction vessels for pretreatment. Among the conditions related with potassium hydroxide combined with urea pretreatment, urea concentration (15 g/L) in this work was lower than 150 g/L (Wang *et al.* 2022), 120 g/L (Wu *et al.* 2023) and 20 g/L (Zahoor *et al.* 2021a; Zahoor *et al.* 2021b) in the four previous reports, which could also reduce input cost of urea for pretreatment in this work. Though potassium hydroxide concentration (45 g/L) in this work was higher than those (20 g/L, 20 g/L) during pretreatment of corn stover (Zahoor *et al.* 2021a) and rice straw (Zahoor *et al.* 2021b), higher solid to liquid ratio (1:5, g/mL) in this work can pretreat more substrate at once and reduce input cost for reaction vessels. Furthermore, solid to liquid ratio (1:5, g/mL) in this work was the most competitive among the reports in Table 3.

Table 3. Comparisons of Pretreatment Conditions along with Recovery and Removal of Cellulosic Components in Lignocellulosic Substrates

Substrates	Pretreatment Conditions	CR	HCR
Sugarcane bagasse	Potassium hydroxide solution 150 g/L, urea 150 g/L, solid to liquid ratio 1:15 (g/mL), 50 °C, 4 h.	93.6%	66.8%
Corn stover	Potassium hydroxide solution 100 g/L, urea 120 g/L, solid to liquid ratio 1:12.5 (g/mL), 80 °C, 60 min.	76.6%	27.4%
Corn stover	Potassium hydroxide solution 20 g/L, urea 20 g/L, solid to liquid ratio 1:10 (g/mL), 30 °C, 2 h.	79.0%	54.3%
Rice straw	Potassium hydroxide solution 20 g/L, urea 20 g/L, solid to liquid ratio 1:15 (g/mL), 70 °C, 3 h.	87.1%	56.2%
Wheat straw	Potassium hydroxide solution (150 g/L), anthraquinone (1 g/L), sodium lignosulfonate (20 g/L), solid to liquid ratio 1:6 (g/mL), 120 °C, 40 min.	80.2%	36.7%
Wheat straw	Sodium hydroxide solution 80 g/L, solid to liquid ratio 1:10 (g/mL), 80 °C, 30.0 min.	85.8%	72.0%

Wheat straw	Ammonium sulfite solution 100 g/L, solid to liquid ratio 1:10 (g/mL), 140 °C, 100 min.	74.7%	64.8%
Wheat straw	Sodium xylene sulfonate solution 300 g/L (pH 3.5), solid to liquid ratio 1:10 (g/mL), 160 °C, 1.0 h.	93.0%	24.0%
Wheat straw	Humic acid solutions 30 g/L, solid to liquid ratio 1:10 (g/mL), at 60°C for 30 min and 180 °C for 40 min.	82.5%	31.3%
Wheat straw	Potassium hydroxide solution 45 g/L, urea 15 g/L, solid to liquid ratio 1:5 (g/mL), 74.0 °C, 50 min.	98.1%	72.6%

CR: Cellulose recovery; HCR: Hemicellulose recovery; LR: Lignin removal

Optimization of Enzymatic Hydrolysis of the Pretreated Wheat Straw

Polysorbate 80, a type of non-ionic surfactant, can act as additive in enzymatic hydrolysis and it can enhance enzymatic digestibility and sugar yields by reducing the unproductive binding of lignin to enzyme, enhancing the interactions between substrate and enzyme, and reinforcing the activity and stability of enzyme (Kamsani *et al.* 2018; Zhang *et al.* 2020a). In addition, the addition of Polysorbate 80 can reduce enzyme loading and reaction time during enzymatic hydrolysis (Ling *et al.* 2021; Zhang *et al.* 2021). Therefore, Polysorbate 80 was added in the reaction mixture and acted as a variable during optimization of enzymatic hydrolysis in this work. As shown in Table 4, variables including enzyme loading, biomass loading, and reaction time had positive and significant effects on glucose and xylose yields. Results indicated that increase of the three variables could promote sugar yields significantly. The three variables also had significant effects on sugar yields from rice straw (Gupta and Parkhey 2014), paddy straw (Manickam *et al.* 2018), and sugarcane tops (Sindhu *et al.* 2014). Enzyme loading and reaction time had significant effects on sugar yields from wheat straw described by Singh and Bishnoi (2012). Enzyme loading and biomass loading had significant effects on sugar yields from sweet sorghum bagasse (Saini *et al.* 2013).

Table 4. Coefficients of Regression for Glucose and Xylose Yields during Optimization of Enzymatic Hydrolysis

Terms	Y_3 (Glucose)	Y_4 (Xylose)
Constant	313.423	97.7333
A (Enzyme loading)	14.331**	7.0333**
B (Biomass loading)	8.951**	8.4033**
C (Polysorbate 80 concentration)	0.864	-0.9767
D (Reaction temperature)	-1.313	-0.8483
E (Reaction pH)	1.977	0.8867
F (Reaction time)	9.613**	6.4583**
Lack of fit	0.107	0.105
R^2	97.93%	98.81%
Adj-R^2	95.86%	97.63%

Outline criterion: 0.05; ** Significant at 1% level

Polysorbate 80, reaction temperature, and pH had insignificant effects on sugar yields in this work. There was no significant effect of Polysorbate 80 on sugar yields during hydrolysis of wheat straw described by Singh and Bishnoi (2012). However, Polysorbate 80 significantly influenced sugar yields from pine foliage (Pandey and Negi 2015) and oil palm empty fruit bunch (Noratiqah *et al.* 2013), respectively. Reaction temperature significantly influenced sugar yields from corn cob described by Garai and Kuma (2013) and pH had a significant effect on hydrolysis of cotton stalk (Du *et al.* 2016). Different effects of variables on sugar yields among different reports were perhaps related with differences of lignocellulosic substrates and hydrolytic enzymes sources. Based on the results of PBD, optimization of enzymatic hydrolysis was carried out at 50 °C, pH 4.8 with 0.4% (w/v) of Polysorbate 80.

Results of the steepest ascent method indicated that glucose and xylose yields reached the plateau while enzyme loading, biomass loading, and reaction time were 8.0 FPU/gds, 145 g/L, and 33.0 h, respectively (Table S5). Sugar yields decreased after the plateau. A high quantity of enzyme loading can increase the rate of transglycosylation reactions along with hydrodynamic instability, improper mixing, and suspension of slurry (Sheng *et al.* 2021). A high quantity of biomass loading can result in poor stirring, enzymatic feedback inhibition by end-products, and decrease of synergistic action of cellulases (Wen *et al.* 2004). Therefore, too high a quantity of enzyme loading and biomass loading were not conducive to sugar yields after the plateau.

According to analysis results of BBD in Table 5, linear terms including X1, X2, and X3 and square terms including X12, X22, and X32 had significant effects on glucose and xylose yields. Interaction terms including X1X2, X1X3, and X2X3 had insignificant effects on glucose and xylose yields. P-values of the models (P = 0.000; P = 0.000) and lack of fit (P = 0.119; P = 0.109) along with high values of R2 (99.4%; 98.7%) and adjusted R2 (98.3%; 96.3%) indicated that the models were adequate to predict glucose and xylose yields.

Table 5. Estimated Regression Coefficients for Glucose and Xylose Yields during Optimization of Enzymatic Hydrolysis

Terms	Y_3 (Glucose)	Y_4 (Xylose)
Constant	607.897	219.380
X_1 (Enzyme loading)	8.216**	-4.752*
X_2 (Biomass loading)	-11.959**	-8.535**
X_3 (Reaction time)	11.030**	-7.530**
$X_1 \times X_1$ (Enzyme loading × enzyme loading)	-17.846**	-24.002**
$X_2 \times X_2$ (Biomass loading × biomass loading)	-66.281**	-15.523**
$X_3 \times X_3$ (Reaction time × reaction time)	-29.378**	-12.857**
$X_1 \times X_2$ (Enzyme loading × biomass loading)	-3.295	0.650
$X_1 \times X_3$ (Enzyme loading × reaction time)	-3.162	2.980
$X_2 \times X_3$ (Biomass loading × reaction time)	-0.177	-1.970
R^2	99.4%	98.7%
Adj-R^2	98.3%	96.3%
Lack of fit	0.119	0.109

Outline criterion: 0.05; * Significant at 5% level; ** Significant at 1% level

Response surface plots and the corresponding counter plots of the interaction effect of each independent variable's pair on glucose and xylose yields were given in Fig. 3 and Fig. 4, respectively, while the other variable was held at zero level. As shown in Fig. 3 (a1, b1), while reaction time (X3) was fixed at its middle level (33.0 h), the optimal regions of enzyme loading (X1) and biomass loading (X2) for glucose yield were 8.4 FPU/gds-9.0 FPU/gds and 136 g/L to144 g/L, respectively. At the same conditions, the optimal regions of enzyme loading (X1) and biomass loading (X2) for xylose yield were 7.2 FPU/gds-7.8 FPU/gds and 136 g/L to 144 g/L, respectively. Figure 3 (a2, b2) indicated that the optimal regions of enzyme loading (X1) and reaction time (X3) for glucose yield were 8.4 FPU/gds-9.0 FPU/gds and 32.0 h to 34.0 h, respectively, while biomass loading (X2) was fixed at its middle level (145 g/L). At the same conditions, the optimal regions of enzyme loading (X1) and reaction time (X3) for xylose yield were 7.2 FPU/gds to 7.8 FPU/gds and 30.0 h - 32.0 h, respectively. Figure 3 (a3, b3) indicated that the optimal regions of biomass loading (X2) and reaction time (X3) for glucose yield were 136 g/L to 144 g/L and 32.0 h to 34.0 h, respectively, while enzyme loading (X1) was fixed at its middle level (8.0 FPU/gds). At the same conditions, the optimal regions of biomass loading (X2) and reaction time (X3) for xylose yield were 136 g/L to 144 g/L and 30.0 h to 32.0 h, respectively.

According to canonical analysis, maximal glucose yield (610.36 mg/gds) could be obtained while enzyme loading, biomass loading and reaction time were 8.45 FPU/gds (X1 = 0.22347), 142.6 g/L (X2 = -0.09600) and 33.9 h (X3 = 0.17599), respectively. Maximal xylose yield (221.85 mg/gds) could be obtained while enzyme loading, biomass loading and reaction time were 7.76 FPU/gds (X1 = -0.12032), 138.5 g/L (X2 = -0.25924), and 31.6 h (X3 = -0.28691), respectively.

The corresponding regression models for glucose and xylose yields were given below in Eq. (11) and Eq. (12):

$$Y3 = 607.897 + 8.216X1 – 11.959X2 + 11.030X3 – 17.846X12 – 66.281X22 – 29.378X32 – 3.295X1X2 – 3.162X1X3 – 0.177X2X3 \quad (11)$$

$$Y4 = 219.380 – 4.752X1 – 8.535X2 – 7.530X3 – 24.002X12 – 15.523X22 – 12.857X32 + 0.650X1X2 + 2.980X1X3 – 1.970X2X3 \quad (12)$$

whereas, Y3 and Y4 were predicted glucose and xylose yields, X1, X2, and X3 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 including enzyme loading 8.1 FPU/gds, biomass loading 141 g/L, and reaction time 32.8 h. Yields of glucose (610.25 mg/gds) and xylose (221.26 mg/gds) could be obtained, which were very in close with the predicted values (610.36 mg/gds, 221.85 mg/gds). Compared with the initial yields of glucose (281.41 mg/gds) and xylose (78.53 mg/gds) under unoptimized conditions, optimization resulted in 1.17-fold increase and 1.82-fold increase for glucose and xylose yields, respectively. In addition, the corresponding cellulose conversion and hemicellulose conversion under the optimized enzymatic hydrolysis conditions were 97.2% and 90.4%, respectively.

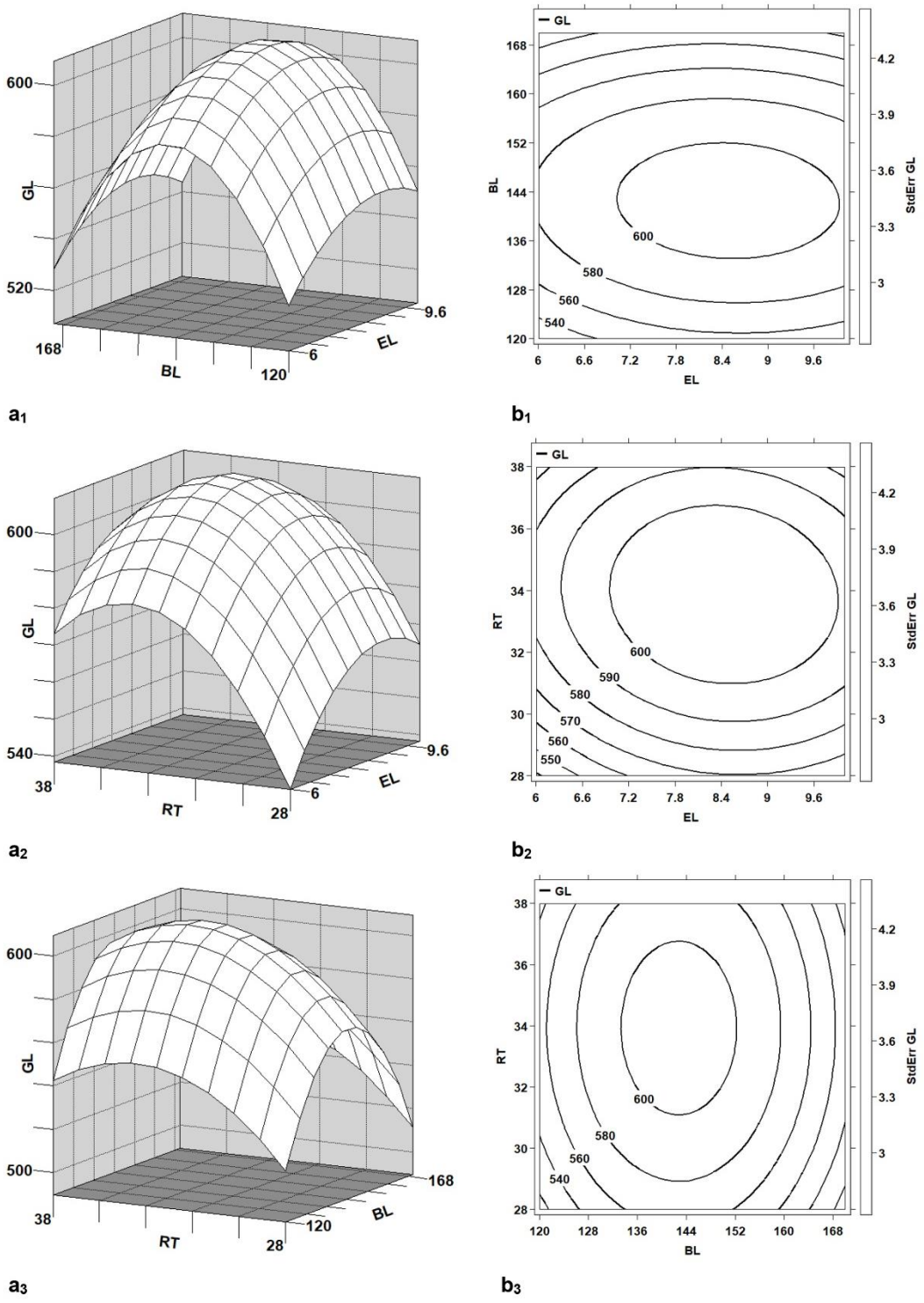

Fig. 3. Response surface plots (a) and contour plots (b) of interaction effect of each independent variable's pair on glucose (Y3). (a1, b1): EL (enzyme loading) and BL (biomass loading); (a2, b2): EL (enzyme loading) and RT (reaction time); (a3, b3): BL (biomass loading) and RT (reaction time)

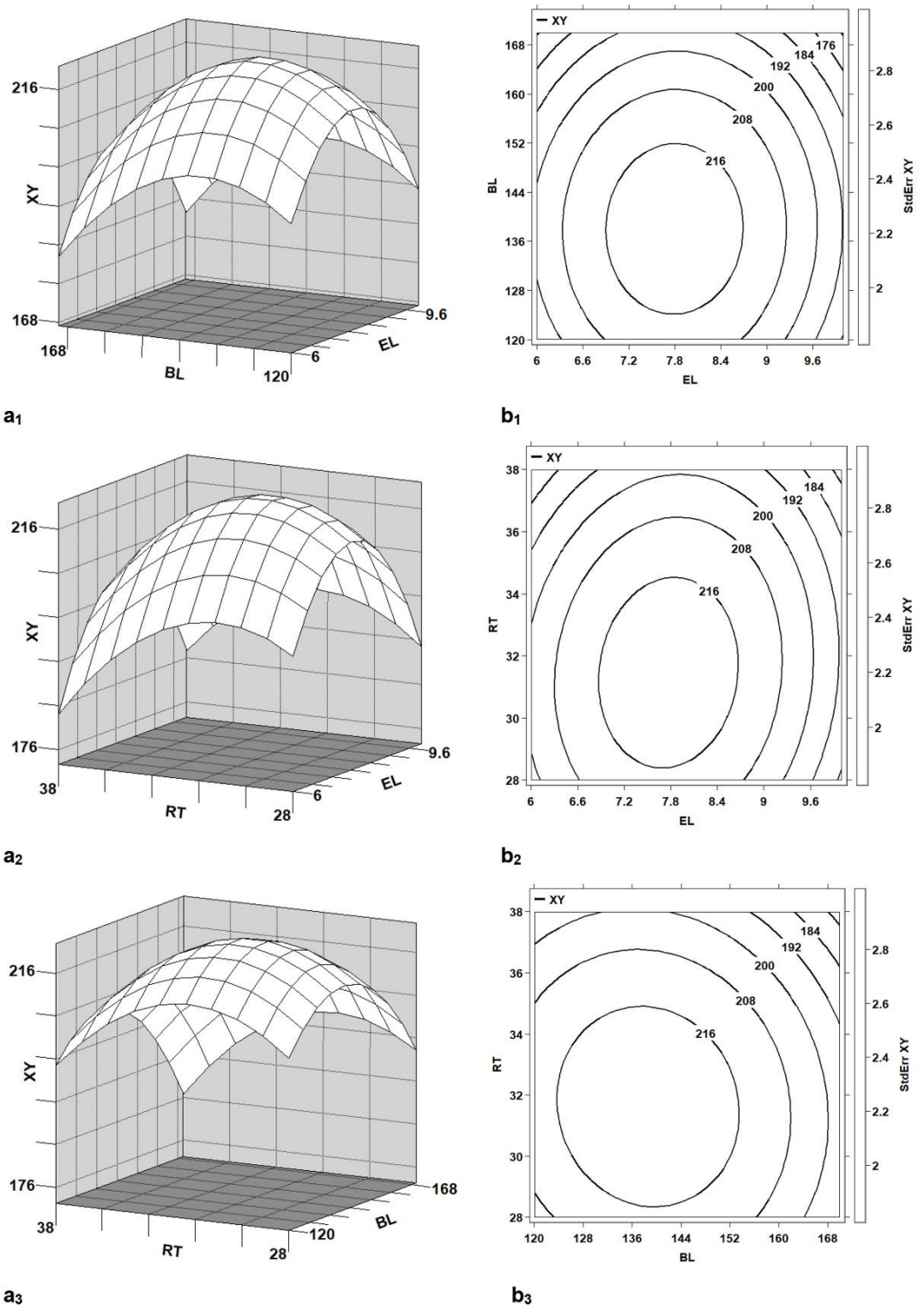

Fig. 4. Response surface plots (a) and contour plots (b) of interaction effect of each independent variable's pair on xylose (Y4). (a1, b1): EL (enzyme loading) and BL (biomass loading); (a2, b2): EL (enzyme loading) and RT (reaction time); (a3, b3): BL (biomass loading) and RT (reaction time)

Comparisons of sugar yields along with enzymatic hydrolysis conditions in this work with those in other previous reports are shown in Table 6. Glucose yield (610.25 mg/gds), xylose yield (221.26 mg/gds), cellulose conversion (97.2%), and hemicellulose conversion (90.4%) in this work were relatively more competitive than those in the other seven reports. Higher cellulose and hemicellulose conversions indicated higher enzymatic digestibility of holocellulose in the pretreated WS in this work. Relatively higher yields of glucose and xylose in this work could provide a basic prerequisite for the future fermentation of bioethanol production.

As shown in Table 6, reaction time (32.8 h) and biomass loading (141 g/L) in this work was more competitive than those in the other seven previous reports. Shorter reaction time and higher biomass loading could reduce input costs of energy and reaction vessels and enhance hydrolysis efficiency. It was obvious that expression of enzyme loading differed from each other among the different reports. For example, enzyme loading was expressed as 10.0 mg/gds glucan by Yuan *et al.* (2018) and FPU/gds combined with CBU/gds by Zheng *et al.* (2018). In the other five previous reports, enzyme loading was expressed as FPU/gds. In addition, cellulases assay conditions in different reports also differed from each other. Therefore, direct comparison of enzyme loading among different reports was not available. Even so, enzyme loading (8.1 FPU/gds) in this work was relatively lower than 41.28 FPU/gds (Sheng *et al.* 2021), 35.0 FPU/gds (Zheng *et al.* 2018), 38.2 FPU/gds (Zhao *et al.* 2017), 20.0 FPU/gds (Momayez *et al.* 2019), 10.0 FPU/gds (Patel *et al.* 2017) and 53.9 FPU/gds (Wu *et al.* 2018) in the other seven previous reports. Lower enzyme loading can reduce cellulases input cost for hydrolysis. In addition, using self-produced cellulases preparation in this work could also reduce input cost of enzymes for enzymatic hydrolysis.

Table 6. Comparisons of Glucose and Xylose Yields of Wheat Straw along with Enzymatic Hydrolysis Conditions

Substrates	Enzyme Sources	Hydrolysis Conditions	GL (mg/g_{ds})	XY (mg/g_{ds})	CC	HC	Ref.
Wheat straw	Cellic@ CTec2 from Sigma-Aldrich	Enzyme loading 41.28 FPU/g_{ds}, biomass loading 41.3 g/L, 72 h.	380.71	-	70.72%	-	Sheng *et al.* 2021
Wheat straw	Cellic CTec2 and β-glucosidase from Novozymes	Enzyme loading 10.0 mg/g_{ds} glucan, biomass loading 100 g/L, 72 h.	606.32	213.53	91.1%	81.7%	Yuan *et al.* 2018
Wheat straw	Mixture of Celluclast 1.5L and cellobiase from Novozym 188	Enzyme loading including 35.0 FPU/g_{ds} and 61.5 CBU/g_{ds}, biomass loading 50 g/L, 72 h.	241.25		87.2%		Zheng *et al.* 2018
Wheat straw	Cellulast 1.5 L® from Novozymes	Enzyme loading 38.2 FPU/g_{ds}, biomass loading 10 g/L, 72 h.	500.33	209.48	89.7	70.9	Zhao *et al.* 2017
Wheat	Cellic CTec2 from	Enzyme loading 20.0 FPU/g_{ds}, biomass	264	104	58.3%	60.0%	Momayez

straw	Novozymes	loading 25 g/L, 48 h.					*et al.* 2019
Wheat straw	Commercial cellulase (SIGMA) and crude cellulases by *Aspergillus niger* ADH-11	Enzyme loading (5.0 FPU/g_{ds} of SIGMA cellulase and 5.0 FPU/g_{ds} of crude cellulase), biomass loading 25 g/L, 72.0 h.	301.2	203.2	53.9	59.6	Patel *et al.* 2017
Wheat straw	Commercial cellulases from Hunan Youtell Biochemical Co., Ltd. Yueyang, China.	Enzyme loading 53.9 FPU/g_{ds}, biomass loading 50 g/L, 72 h.	484.5	101.7	78.3%	54.1%	Wu *et al.* 2018
Wheat straw	*Aspergillus niger* HQ-1	Enzyme loading 8.1 FPU/g_{ds}, biomass loading 141 g/L, 32.8 h.	610.25	221.26	97.2%	90.4%	This work

GL: Glucose; XY: Xylose; CC: Cellulose conversion; HC: Hemicellulose conversion; CBU: Cellubioase activity unit

CONCLUSIONS

1. This is the first report about optimization of potassium hydroxide combined with urea pretreatment and the following enzymatic hydrolysis of the pretreated wheat straw (WS) using response surface methodology (RSM) for improving sugar yields. The optimized pretreatment conditions could result in considerable cellulose recovery (98.1%), hemicellulose recovery (72.6%), and lignin removal (75.8%).
2. Under the premise of adopting relatively higher solid to liquid ratio (1:5, g/mL) during pretreatment, using moderate concentration of potassium hydroxide (45 g/L) and lower concentration of urea (15 g/L) can reduce input cost of reagents for pretreatment. Relatively higher yields of glucose (610.25 mg/g_{ds}) and xylose (221.26 mg/g_{ds}) along with higher cellulose conversion (97.2%) and hemicellulose conversion (90.4%) indicated that the optimized enzymatic hydrolysis conditions could enhance enzymatic digestibility of holocellulose in the pretreated WS.
3. Adoption of higher solid to liquid ratio (1:5, g/mL) during pretreatment and adoption of higher biomass (141 g/L), shorter reaction time (32.8 h) and self-produced cellulases preparation during hydrolysis in this work could reduce input cost for reaction vessels and enzymes and enhance efficiency for sugar yields.

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Authors' Contributions

Hui Zhang conceived and designed the total experiments. Hui Zhang performed the experiments of optimization of pretreatment and analyzed the data. Junhui Wu performed the experiments including optimization of enzymatic hydrolysis and cellulases extraction by *A. niger* HQ-1 and analyzed the data. Hui Zhang wrote, reviewed, and edited the manuscript.

Data Availability

All data generated or analyzed during this study are included in the article and the Supplementary Materials, those are available from the corresponding author upon reasonable request.

Competing Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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APPENDIX
Supplementary Information

Table S1. Codes and Levels of Five Variables in Plackett-Burman Design (PBD) along with Glucose and Xylose Yields during Optimization of Pretreatment

Trials	A	B	C	D	E	Y_1	Y_2
1	1 (15)	-1	1 (50)	-1 (20)	-1 (1:5)	141.37 ± 1.86	28.42 ± 0.56
2	1	1 (15)	-1 (40)	1 (30)	-1	140.14 ± 1.46	27.71 ± 0.40
3	-1 (8)	1	1	-1	1 (1:4)	119.52 ± 1.59	22.94 ± 0.37
4	1	-1	1	1	-1	154.56 ± 2.28	35.39 ± 0.67
5	1	1	-1	1	1	137.39 ± 1.93	26.59 ± 0.42
6	1	1	1	-1	1	140.48 ± 1.72	29.23 ± 0.49
7	-1	1	1	1	-1	133.01 ± 1.68	27.94 ± 0.48
8	-1	-1	1	1	1	130.36 ± 1.73	28.21 ± 0.51
9	-1	-1	-1	1	1	122.17 ± 1.58	20.77 ± 0.33
10	1	-1	-1	-1	1	118.05 ± 1.79	21.20 ± 0.38
11	-1	1	-1	-1	-1	107.33 ± 1.63	16.41 ± 0.25
12	-1	-1 (8)	-1	-1	-1	104.29 ± 1.58	15.87 ± 0.26
13	0	0	0	0	0	128.82 ± 1.73	27.11 ± 0.41
14	0	0	0	0	0	126.57 ± 1.51	27.56 ± 0.39
15	0	0	0	0	0	127.99 ± 1.83	27.34 ± 0.49

A: Potassium hydroxide concentration (g/L); B: Urea concentration (g/L); C: Pretreatment temperature (°C); D: Pretreatment time (min); E: Solid to liquid ratio (g/mL); Y_1: Glucose (mg/g_{ds}); Y_2: Xylose (mg/g_{ds})

Table S2. Design of the Steepest Ascent Method along with Glucose and Xylose Yields during Optimization of Pretreatment

Steps	x_1 (Potassium hydroxide concentration, g/L)	x_2 (Pretreatment temperature, °C)	x_3 (Pretreatment time, min)	Y_1 (Glucose, mg/g_{ds})	Y_2 (Xylose, mg/g_{ds})
1	15	55	30	166.67 ± 2.86	39.05 ± 0.65
2	30	65	40	212.33 ± 3.64	59.14 ± 0.96
3	45	75	50	277.97 ± 4.58	77.34 ± 1.29
4	60	85	60	233.90 ± 3.52	48.13 ± 0.75
5	75	95	70	177.53 ± 3.01	30.42 ± 0.61

Table S3. Codes and Levels of Variables in CCD along with Glucose and Xylose Yields during Optimization of Pretreatment

Runs	x_1 (Potassium hydroxide concentration, g/L)	x_2 (Pretreatment temperature, °C)	x_3 (Pretreatment time, min)	Y_1 (Glucose, mg/g_{ds})	Y_2 (Xylose, mg/g_{ds})
1	-1 (30)	-1 (65)	-1 (40)	212.43 ± 3.03	59.06 ± 0.84
2	1 (60)	-1	-1	223.15 ± 3.56	54.02 ± 0.73
3	-1	1 (85)	-1	205.17 ± 3.14	54.76 ± 0.81
4	1	1	-1	215.79 ± 3.22	52.55 ± 0.78
5	-1	-1	1 (60)	223.99 ± 3.69	55.99 ± 0.84
6	1	-1	1	240.28 ± 3.87	51.17 ± 0.76
7	-1	1	1	217.73 ± 3.55	51.96 ± 0.79
8	1	1	1	233.32 ± 3.82	48.18 ± 0.61
9	-1.682 (19.7)	0 (75)	0 (50)	201.12 ± 3.15	58.49 ± 0.81
10	1.682 (70.2)	0	0	220.65 ± 3.87	48.69 ± 0.83
11	0	-1.682 (58)	0	228.24 ± 4.01	60.49 ± 0.98
12	0 (45)	1.682 (92)	0	217.84 ± 3.76	55.26 ± 0.72
13	0	0	-1.682 (33)	225.05 ± 3.28	55.48 ± 0.92
14	0	0	1.682 (67)	240.76 ± 3.80	46.34 ± 0.73
15	0	0	0	277.76 ± 4.28	78.43 ± 1.22
16	0	0	0	280.86 ± 4.78	77.73 ± 1.18
17	0	0	0	277.87 ± 4.39	77.03 ± 1.27
18	0	0	0	280.97 ± 4.71	76.72 ± 1.21
19	0	0	0	277.88 ± 4.60	76.88 ± 1.18
20	0	0	0	281.05 ± 4.53	78.43 ± 1.25

Table S4. Codes and Levels of Six Variables and Plackett-Burman Design (PBD) along with Glucose and Xylose Yields during Optimization of Enzymatic Hydrolysis

Trials	A	B	C	D	E	F	Y_3	Y_4
1	1 (4.0)	-1 (70)	1 (0.4)	-1 (50)	-1 (4.4)	-1 (12.0)	304.25 ± 4.41	86.78 ± 1.23
2	1	1 (95)	-1 (0.2)	1 (55)	-1	-1	323.87 ± 4.39	105.99 ± 1.60
3	-1 (2.0)	1	1	-1	1 (4.8)	-1	306.21 ± 4.98	92.12 ± 1.47
4	1	-1	1	1	-1	1 (18.0)	330.03 ± 5.21	100.34 ± 1.53
5	1	1	-1	1	1	-1	325.96 ± 5.17	107.69 ± 1.41
6	1	1	1	-1	1	1	350.56 ± 5.96	122.65 ± 1.71
7	-1	1	1	1	-1	1	314.98 ± 4.87	103.18 ± 1.33
8	-1	-1	1	1	1	-1	279.69 ± 3.56	75.47 ± 1.17
9	-1	-1	-1	1	1	1	298.13 ± 4.25	88.64 ± 1.31
10	1	-1	-1	-1	1	1	331.85 ± 5.10	105.15 ± 1.38
11	-1	1	-1	-1	-1	1	312.66 ± 4.92	105.19 ± 1.33
12	-1	-1	-1	-1	-1	-1	282.88 ± 4.02	79.60 ± 1.28
13	0	0	0	0	0	0	312.56 ± 4.46	99.41 ± 1.37
14	0	0	0	0	0	0	309.75 ± 4.39	100.33 ± 1.32
15	0	0	0	0	0	0	311.91 ± 4.58	100.82 ± 1.27

A: Enzyme loading (FPU/g_{ds}); B: Biomass loading (g/L); C: Polysorbate 80 concentration (%, w/v); D: Reaction temperature (°C); E: Reaction pH; F: Reaction time (h); Y_3: Glucose (mg/g_{ds}); Y_4: Xylose (mg/g_{ds})

Table S5. Design of the steepest ascent method along with glucose and xylose yields during optimization of enzymatic hydrolysis

Steps	X_1 (Enzyme loading, (FPU/g_{ds})	X_2 (Biomass loading, g/L)	X_3 (Reaction time, h)	Y_3 (Glucose, mg/g_{ds})	Y_4 (Xylose, mg/g_{ds})
1	4.0	95	23.0	392.74 ± 5.26	145.88 ± 2.03
2	6.0	120	28.0	483.64 ± 6.32	180.06 ± 2.62
3	8.0	145	33.0	607.43 ± 6.62	221.34 ± 2.91
4	10.0	170	38.0	473.67 ± 5.87	155.26 ± 2.07
5	12.0	195	43.0	372.22 ± 5.02	115.88 ± 2.31

Table S6. Codes and levels of variables and BBD along with glucose and xylose yields during optimization of enzymatic hydrolysis

Runs	X_1	X_2	X_3	Y_3	Y_4
1	-1 (6.0)	-1 (120)	0 (33.0)	526.04 ± 6.21	194.17 ± 2.84
2	1 (10.0)	-1	0	549.73 ± 6.71	185.66 ± 2.54
3	-1	1 (170)	0	504.40 ± 6.28	172.75 ± 2.91
4	1	1	0	514.91 ± 7.14	166.84 ± 2.19
5	-1	0 (145)	-1 (28.0)	535.40 ± 7.36	200.73 ± 3.25
6	1	0	-1	557.49 ± 8.01	182.97 ± 3.08
7	-1	0	1 (38.0)	570.18 ± 8.28	176.11 ± 2.61
8	1	0	1	579.62 ± 7.85	170.27 ± 2.73
9	0 (8.0)	-1	-1	514.03 ± 6.89	201.77 ± 2.91
10	0	1	-1	494.78 ± 6.03	191.69 ± 2.98
11	0	-1	1	530.05 ± 7.34	194.25 ± 2.79
12	0	1	1	510.09 ± 7.36	176.29 ± 2.82
13	0	0	0	610.02 ± 8.19	219.64 ± 3.53
14	0	0	0	608.22 ± 8.17	217.78 ± 3.48
15	0	0	0	605.45 ± 8.36	220.72 ± 3.46

X_1: Enzyme loading (FPU/g_{ds}); X_2: Biomass loading (g/L); X_3: Reaction time (h); Y_3: Glucose (mg/g_{ds}); Y_4: Xylose (mg/g_{ds})