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# Optimization of Alkaline Hydrogen Peroxide Pretreatment and Enzymatic Hydrolysis of Wheat Straw for Enhancing Sugar Yields

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**Abstract:** Optimization of alkaline hydrogen peroxide (AHP) pretreatment and enzymatic hydrolysis of wheat straw (WS) was carried out to enhance fermentable sugar yields with the use of glucose and xylose yields from the pretreated WS as responses. In the first step, variables including temperature, hydrogen peroxide concentration and time during pretreatment were detected to have significant effects on the sugar yields. The results indicate that maximal sugar yields could be obtained while the WS was pretreated using 71 g/L hydrogen peroxide solution with 200 g/L of solid loading at 50 °C for 7.6 h. The corresponding cellulose recovery, hemicellulose recovery and lignin removal were 97.5%, 84.3% and 75.0%, respectively. In the second step, enzymatic hydrolysis of the pretreated WS was optimized. The results show that the reaction time, enzyme loading and biomass loading during enzymatic hydrolysis also had significant effects on the sugar yields. The final maximum yields of glucose (552.7 mg/$g_{ds}$ (mg/g dry substrate)) and xylose (223.6 mg/$g_{ds}$) could be obtained while enzymatic hydrolysis was carried out at 50 °C for 37.0 h using 10.8 FPU/$g_{ds}$ (filter paper activity unit per gram dry substrate) of enzyme loading, 88 g/L of biomass loading and 0.3% (*w*/*v*) of Tween-80. The corresponding cellulose conversion and hemicellulose conversion were 94.0% and 83.5%, respectively.

**Keywords:** wheat straw; enzymatic hydrolysis; alkaline hydrogen peroxide pretreatment; optimization; response surface methodology



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## 1. Introduction

Lignocellulosic biomass is a long-term feedstock with immense potential for the production of bioethanol, which can alleviate the global energy crisis and environmental quality deterioration [1]. Lignocellulosic substrates mainly contain three types of components, namely, cellulose, hemicellulose and lignin. Holocellulose, including cellulose and hemicellulose, can be hydrolyzed to sugar monomers, such as glucose and xylose, which can be utilized to produce ethanol during the fermentation process [2]. However, the presence of lignin can hinder the utilization of holocellulose by enzymes. Therefore, pretreatment is necessary to break down the lignocellulosic structure, reduce the lignin content, reduce the crystallinity of cellulose, increase the accessible surface area for the action of enzymes and enhance the enzymatic digestibility of holocellulose [3]. Among the different pretreatment methods, alkaline pretreatment can dissolve most of the lignin and the various produced uronic acid substitutions, which can inhibit the accessibility of cellulose during enzymatic hydrolysis [4]. Hydrogen peroxide is commonly used in the pulping and bleaching industries. It can react with the chromophores present in lignin, which results in the bleaching of substrates [5]. In fact, an alkali reagent can be combined with hydrogen peroxide, which is named alkaline hydrogen peroxide (AHP) pretreatment. AHP pretreatment has many favorable properties, such as mild temperature and pressure, readily available reagents with low toxicity and low environmental impact, downstream

processing compatibility and high efficiency [6]. In previous reports, AHP was adopted to pretreat some lignocellulosic substrates, such as corn stover [7], rice husk [8] and sugarcane bagasse [9], and it was shown to be an effective pretreatment method. Under alkaline conditions, especially close to pH 11.5, hydrogen peroxide will dissociate to produce hydroperoxyl anion ($HOO^-$), which is responsible for the corresponding carbonyl and ethylene groups' oxidative reactions and is an initiator for radical formation. The hydroperoxyl anions ($HOO^-$) will react with hydrogen peroxide to produce the highly reactive hydroxyl radical ($HO\cdot$) and superoxide anion radical ($O_2^{-}\cdot$), which can cause lignin to be oxidized and depolymerized into low-molecular-weight fragments [10]. It is noteworthy that hydroperoxyl anions ($HOO^-$) are more selective than radicals to attack lignin rather than holocellulose, which can reduce the degradation of holocellulose. Furthermore, the action of hydrogen peroxide can be optimized with the use of modeling [11]. Even so, the optimization of an AHP pretreatment is also crucial to further reduce the input cost of pretreatment, retain a larger amount of holocellulose and realize higher delignification simultaneously.

During the enzymatic hydrolysis of the pretreated lignocellulosic substrates for fermentable sugar production, cellulases and xylanases are always used. Cellulose can be hydrolyzed via the synergy actions of β-1, 4-endoglucanases (EC 3.2.1.4); β-1, 4-exoglucanases, which are also named cellobiohydrolases (EC 3.2.1.91); and β-D-glucosidases (EC 3.2.1.21). Hemicellulose (xylan) can be hydrolyzed through the coordination of endoxylanases (EC 3.2.1.8) and β-xylosidases (EC 3.2.1.37). No matter what type of enzymes are used, enzymatic activities can be influenced by factors including temperature, pH, enzyme loading and biomass loading. Therefore, it is necessary to optimize the enzymatic hydrolysis of the pretreated substrates to enhance sugar yields and enzymatic hydrolysis efficiency. In addition, the adoption of inexpensive enzymes and substrates during enzymatic hydrolysis can reduce the input cost of hydrolysis.

Wheat straw (WS) is an abundant and cheap lignocellulosic residue in North China, which makes it possible to be applied to fermentable sugar production at a large scale. Response surface methodology (RSM) is a statistical modeling technique that can establish a multivariate equation based on quantitative data in the designed experiments [12,13]. With the use of RSM, the objective of this work was to optimize the AHP pretreatment of WS and enzymatic hydrolysis of the pretreated WS to enhance fermentable sugar yields for bioethanol production.

## 2. Materials and Methods

### *2.1. Biomass and Chemicals*

WS was obtained from a nearby farm in Liaocheng, Shandong Province, China. The WS was cut to 1–2 cm in length without grinding and was dried at 85 °C until a constant weight was achieved. Sodium hydroxide (AR), hydrogen peroxide (30%, *w*/*w*, AR), D-glucose (AR), acetic acid (AR), sodium acetate (AR) and Tween-80 (CP) were purchased from Xilong Scientific Co., Ltd., Shantou, China. D-xylose (BR) was purchased from Sinopharm Chemical Reagent Co., Ltd., Shanghai, China.

### *2.2. Enzymes Production, Extraction and Activities Assays*

The cellulase preparation was achieved with *Aspergillus niger* HQ-1 (accession number of ITS sequence: HQ891869) in a laboratory according to methods described in our previous report [14] and the Supplementary Materials. Cellulase and xylanase activities were also assayed and defined according to the methods in the Supplementary Materials. After the optimization of the cellulases extraction (Tables S1–S6), the 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 obtained.

### 2.3. Optimization of Alkaline Hydrogen Peroxide Pretreatment

With the use of 250 mL triangle flasks, pretreatment was carried out by soaking WS in 100 mL hydrogen peroxide solution at pH 11.5, which was adjusted using a 5.0 M sodium hydroxide solution. Different pretreatment conditions were designed based on the Plackett–Burman design (PBD), and five variables, namely, pretreatment temperature, solid loading, hydrogen peroxide concentration, agitation speed and pretreatment time, were adopted in the PBD (Table S7). Two levels, namely, low level (−1) and high level (+1), of the tested variables—pretreatment time (25 °C, 35 °C), solid loading (100, 200, g/L), hydrogen peroxide concentration (10, 25, g/L), agitation speed (100 rpm, 150 rpm) and pretreatment time (3.0 h, 5.0 h)—were adopted. The pretreated WS was washed and filtered with double-distilled water till neutrality. After being dried in an oven at 80 °C for 24 h, the pretreated WS was hydrolyzed under initial conditions (Section 2.5.1). The produced glucose and xylose yields from the pretreated WS were adopted as responses for the optimization of pretreatment. Based on the analysis of the PBD, significant variables for pretreatment, including temperature, hydrogen peroxide concentration and time, were screened and their optimal regions were detected using the method of steepest ascent (Table S8). Finally, the optimal values of the variables were determined using a Box–Behnken design (BBD) (Table S9). Each variable was investigated at three levels, namely, low level (−1), middle level (0) and high level (+1). Twelve experimental trials with different combinations of three variables, namely, temperature (43 °C, 49 °C and 55 °C), hydrogen peroxide concentration (55, 70 and 85 g/L) and time (6.9 h, 7.5 h and 8.1 h), along with three replication trials of the center points were designed. All experiments were performed in triplicate and the obtained data were used with the least squares method to fit the second-order polynomial model, as given by the following Equation (1):

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

in which $Y$ is the predicted response, $\beta_0$ is the intercept term, $\beta_i$ is the linear coefficient, $\beta_{ii}$ is the squared coefficient and $\beta_{ij}$ is the interaction coefficient.

### 2.4. Chemical Composition Analyses and Calculations

The contents of cellulose, hemicellulose and lignin in the obtained WS pretreated under the optimized conditions and raw WS were determined three times according to the methods described by Aravantinos-Zafiris et al. (1994) [15]. Solid recovery, cellulose recovery, hemicellulose recovery and lignin removal were calculated based on the following equations:

$$\text{Solid recovery (\%)} = (DW_1/DW_0) \times 100\% \quad (2)$$

where $DW_1$ is the dry weight of WS recovered after pretreatment (g) and $DW_0$ is the dry weight of WS before pretreatment (g);

$$\text{Cellulose recovery (\%)} = (RC_{PT\text{-}WS} \times SR/C_{WS}) \times 100\% \quad (3)$$

where SR is the solid recovery after pretreatment and $C_{WS}$ and $RC_{PT\text{-}WS}$ are the amounts of cellulose in raw and pretreated WS expressed in g/g, respectively;

$$\text{Hemicellulose recovery (\%)} = [RHC_{PT\text{-}WS} \times SR/HC_{WS}] \times 100\% \quad (4)$$

where SR is the solid recovery after pretreatment and $HC_{WS}$ and $RHC_{PT\text{-}WS}$ are the amounts of hemicellulose in raw and pretreated WS expressed in g/g, respectively;

$$\text{Lignin removal (\%)} = [(L_{WS} - RL_{PT\text{-}WS} \times SR)/L_{WS}] \times 100\% \quad (5)$$

where SR is the solid recovery after pretreatment and $L_{WS}$ and $RL_{PT\text{-}WS}$ are the amounts of lignin in raw and pretreated WS expressed in g/g, respectively.

### *2.5. Initial Enzymatic Hydrolysis Conditions and Optimization of Enzymatic Hydrolysis*

#### 2.5.1. Initial Enzymatic Hydrolysis Conditions

At first, the pretreated WS was incubated in 100 mL sodium acetate buffer (50 mM, pH 4.4) containing 0.3% (*w*/*v*) Tween-80 at 20 g/L of biomass loading in 250 mL Erlenmeyer flasks. The antibiotics tetracycline (40 μg/mL) and cycloheximide (30 μg/mL) were supplemented in the mixture to prevent microbial contamination. Then, the enzyme preparation was added mainly according to 5.0 FPU/$g_{ds}$ (filter paper activity unit per gram dry substrate) of enzyme loading in the reaction mixture. Enzymatic hydrolysis was performed at 50 °C and 120 rpm for 26.0 h. Finally, the residues were separated via centrifugation at 13,980× *g* (10,000 rpm) for 10 min and the obtained supernatant was collected to determine the monosaccharide contents. The monosaccharides (glucose and xylose) were determined via HPLC (Agilent 1200 series; Hewlett-Packard, Palo Alto, CA, USA) equipped with an Aminex HPX-87H ion exclusion column (300 × 7.8 mm; Bio-Rad Laboratories, Hercules, CA, USA) and a differential refraction detector using 5.0 mM $H_2SO_4$ as an eluent at a flow rate of 0.6 mL/min. The yields of glucose and xylose were expressed as milligrams per gram of dry substrate (mg/$g_{ds}$). The control of each reaction mixture was performed by replacing the active crude enzymes with heat-inactivated (100 °C, 10 min) enzymes.

#### 2.5.2. Optimization of Enzymatic Hydrolysis

In the first step, PBD was also adopted to investigate the effects of the variables during enzymatic hydrolysis (Table S10). Two levels, namely, low level (−1) and high level (+1), of six variables, namely, reaction time (26.0 h, 30.0 h), reaction temperature (50.0 °C, 55.0 °C), enzyme loading (3.0 FPU/$g_{ds}$, 7.0 FPU/$g_{ds}$), reaction pH (4.4, 4.8), biomass loading (20, 40 g/L) and Tween-80 concentration (0.3%, 0.5% *w*/*v*), were adopted. The glucose and xylose yields were determined and regarded as responses during the optimization. Based on the analysis of the PBD, significant variables, including the reaction time, enzyme loading and biomass loading, were measured and their optimal regions were investigated using the method of steepest ascent (Table S11). Lastly, a central composite design (CCD) with twenty trials was employed to determine the optimal values of the significant variables (Table S12). Five levels, namely, −1.682, −1, 0, +1 and +1.682 (coded values), of the significant variables, namely, reaction time (29.3 h, 32.0 h, 36.0 h, 40.0 h and 42.7 h), enzyme loading (4.95 FPU/$g_{ds}$, 7.0 FPU/$g_{ds}$, 10.0 FPU/$g_{ds}$, 13.0 FPU/$g_{ds}$ and 15.05 FPU/$g_{ds}$) and biomass loading (56.4, 70, 90, 110 and 123.6 g/L), were designed. All experiments were performed in triplicate and yields of glucose and xylose were used as responses. The obtained data were analyzed using the least squares method to fit the second-order polynomial model given by the following Equation (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 $Y$ is the predicted response; $X_1$, $X_2$ and $X_3$ are the coded variables; $\beta_0$ is the intercept; $\beta_1$, $\beta_2$ and $\beta_3$ are the linear coefficients; $\beta_{11}$, $\beta_{22}$ and $\beta_{33}$ are the squared coefficients; and $\beta_{12}$, $\beta_{13}$ and $\beta_{23}$ are the interaction coefficients.

### *2.6. Calculations for Enzymatic Hydrolysis*

The cellulose conversion and hemicellulose conversion were calculated according to the following equations:

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

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

where $M_g$ is the amount of glucose from the pretreated WS (mg/$g_{ds}$); $M_c$ is the amount of cellulose in the pretreated WS (mg/$g_{ds}$); $M_x$ is the amount of xylose from the pretreated WS (mg/$g_{ds}$); $M_h$ is the amount of hemicellulose in the pretreated WS (mg/$g_{ds}$); and 0.9 and 0.88 are the conversion factors of glucose and xylose, respectively.

### 2.7. 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 ($p \leq 0.05$).

## 3. Results and Discussions

### 3.1. Optimization of Alkaline Hydrogen Peroxide Pretreatment of Wheat Straw

The degradation of holocellulose occurs along with delignification, which indicates that holocellulose recovery and lignin removal are not suitable responses during pretreatment. The main aim of pretreatment is to enhance the enzymatic digestibility of substrates for higher yields of fermentable sugar during the following enzymatic hydrolysis. Therefore, glucose and xylose yields during enzymatic hydrolysis of the pretreated WS were more suitable responses for the pretreatment optimization. It was reported that a mild temperature (<100 °C) can achieve significant delignification and high glucan enrichment during AHP pretreatment [16,17]. In addition, too high of a temperature can result in more carboxylic acids, such as acetic acid, because acetyl groups are removed from hemicellulose during pretreatment. The produced acids can markedly change the pH in a reaction mixture from pH 11.9 to pH 5.63 [10]. During AHP pretreatment, pH 11.5 is always adopted, as hydrogen peroxide will dissociate to produce the hydroperoxyl anion ($HOO^-$), which is responsible for delignification [10]. Therefore, a mild temperature (<100 °C) and pH 11.5 were prioritized in this work.

According to the analysis of the PBD (Table 1), the pretreatment temperature, hydrogen peroxide concentration and pretreatment time had significant effects on the sugar production. The temperature and time also have significant effects during the pretreatment of corn stover [18] and wheat straw [19]. The temperature had a positive effect on the sugar production, as a higher temperature could enhance the reaction rate constant. A high temperature can also improve the pretreatment efficiency of oil palm trunks [20] and corn stover [21,22], respectively. In addition, the hydrogen peroxide concentration also had a positive effect on sugar production, as a higher concentration of it could improve the delignification degree. The other factors, including the solid loading and agitation speed, had insignificant effects on the sugar yields. The optimization of the significant factors was carried out in subsequent experiments by adopting a lower agitation speed (100 rpm) and higher solid loading (200 g/L).

**Table 1.** Coefficients of regression for glucose and xylose yields during pretreatment optimization.

| Terms | $Y_1$ (Glucose, mg/$g_{ds}$) | $Y_2$ (Xylose, mg/$g_{ds}$) |
|---|---|---|
| Constant | 88.437 | 31.268 |
| A: Pretreatment temperature | 9.848 $^{SS}$ | 2.527 $^{SS}$ |
| B: Solid loading | 0.067 | 0.259 |
| C: Hydrogen peroxide concentration | 7.527 $^{SS}$ | 1.731 $^{SS}$ |
| D: Agitation speed | −0.632 | −0.102 |
| E: Pretreatment time | 6.895 $^{SS}$ | 2.964 $^{SS}$ |
| $R^2$ | 98.07% | 96.79% |
| Adj-$R^2$ | 96.63% | 94.38% |
| Lack of fit | 0.118 | 0.138 |

Outline criterion: 0.05; $^{SS}$ significant at 1% level.

The results indicate that both the glucose and xylose yields reached a plateau while the pretreatment temperature, hydrogen peroxide concentration and pretreatment time were 49.0 °C, 70 g/L and 7.5 h, respectively. Too low levels of the three factors can result in lower delignification and too high levels can degrade more holocellulose, along with produce higher delignification, which is also not conducive to fermentable sugar yields. The statistical analysis of the BBD (Table 2) indicated that the linear terms, namely, $x_1$, $x_2$ and $x_3$, and the square terms, namely, ${x_1}^2$, ${x_2}^2$ and ${x_3}^2$, had significant effects on the glucose

and xylose yields. The interaction terms, namely, $x_1x_2$, $x_1x_3$ and $x_2x_3$, had insignificant effects on the glucose yield, whereas only $x_1x_3$ had a significant effect on the xylose yield. $F$ values of the models ($F$ = 86.69, $F$ = 94.83) and lack of fit ($F$ = 6.23, $F$ = 7.83), along with $p$-values of the models ($p$ = 0.000, $p$ = 0.000) and lack of fit ($p$ = 0.141, $p$ = 0.115), indicate that the obtained experimental data for glucose and xylose yields were a good fit with the models, respectively. The values of $R^2$ (99.4%, 99.4%) and adjusted $R^2$ (98.2%, 98.4%) also indicate the accuracy of the models.

**Table 2.** Estimated regression coefficients for glucose and xylose yields during pretreatment optimization.

| Terms | Glucose ($Y_1$) | Xylose ($Y_2$) |
|---|---|---|
| Constant | 233.213 | 91.1433 |
| Pretreatment temperature ($x_1$) | 5.165 $^{SS}$ | 2.7813 $^{SS}$ |
| Hydrogen peroxide concentration ($x_2$) | 3.090 $^{S}$ | 2.6913 $^{SS}$ |
| Pretreatment time ($x_3$) | 3.280 $^{S}$ | 3.3475 $^{SS}$ |
| Pretreatment temperature × pretreatment temperature ($x_1 \times x_1$) | −30.647 $^{SS}$ | −9.3917 $^{SS}$ |
| Hydrogen peroxide concentration × hydrogen peroxide concentration ($x_2 \times x_2$) | −18.622 $^{SS}$ | −8.0867 $^{SS}$ |
| Pretreatment time × pretreatment time ($x_3 \times x_3$) | −28.842 $^{SS}$ | −5.7692 $^{SS}$ |
| Pretreatment temperature × hydrogen peroxide concentration ($x_1 \times x_2$) | 0.765 | −0.6000 |
| Pretreatment temperature × pretreatment time ($x_1 \times x_3$) | 0.060 | −2.3225 $^{SS}$ |
| Hydrogen peroxide × pretreatment time ($x_2 \times x_3$) | −0.425 | −1.1625 |
| $R^2$ | 99.4% | 99.4% |
| Adj-$R^2$ | 98.2% | 98.4% |
| Lack of fit | 0.141 | 0.115 |

Outline criterion: 0.05; $^{S}$ significant at 5% level; $^{SS}$ significant at 1% level.

Three-dimensional response surface plots and the corresponding contour plots were used to demonstrate the combined effects of each variable's pair on yields of glucose and xylose, as shown in Figures 1 and 2, respectively. As shown in Figures 1($a_1$,$b_1$) and 2($a_1$,$b_1$), while the time was fixed at its middle level (7.5 h), the optimal regions of the temperature and hydrogen peroxide concentration for the yields of glucose and xylose were 48 °C–50 °C and 70 g/L–75 g/L, respectively. As shown in Figure 1($a_2$,$b_2$), while the hydrogen peroxide concentration was set to be its middle level (70 g/L), the optimal regions of the temperature and time for the glucose yield were 48 °C–50 °C and 7.4 h–7.6 h, respectively. Under the same conditions in Figure 2($a_2$,$b_2$), the optimal regions of the temperature and time for the xylose yield were 48 °C–50 °C and 7.6 h–7.8 h, respectively. As shown in Figure 1($a_3$,$b_3$), while the temperature was fixed at its middle level (49 °C), the optimal regions of the hydrogen peroxide concentration and time for the glucose yield were 70 g/L–75 g/L and 7.4 h–7.6 h, respectively. Under the same conditions in Figure 2($a_3$,$b_3$), the optimal regions of the hydrogen peroxide concentration and time for the xylose yield were 70 g/L–75 g/L and 7.6 h–7.8 h, respectively.

According to canonical analysis, the maximal glucose (233.7 mg/$g_{ds}$) could be obtained while WS was pretreated using 71 g/L hydrogen peroxide solution ($x_2$ = 0.084) at 49.5 °C ($x_1$ = 0.085) for 7.5 h ($x_3$ = 0.056). The maximal xylose (91.9 mg/$g_{ds}$) could be obtained while WS was pretreated using 72 g/L hydrogen peroxide solution ($x_2$ = 0.14405) at 49.6 °C ($x_1$ = 0.11218) for 7.7 h ($x_3$ = 0.25303).

To validate the predicted conditions, the WS was pretreated using 71 g/L hydrogen peroxide solution at 50 °C for 7.6 h. After the enzymatic hydrolysis, the yields of glucose (234.2 mg/$g_{ds}$) and xylose (92.5 mg/$g_{ds}$) (average of three replicates) were obtained, which were close to the predicted values from the models.

In addition, the contents of cellulose, hemicellulose and lignin in the raw WS (dry weight 20.00 g) were 43.52%, 22.41% and 9.80%, respectively. After pretreatment, the contents of cellulose, hemicellulose and lignin in the pretreated WS (dry weight 16.04 g) were 52.92%, 23.56% and 3.05%, respectively. After the calculation, the pretreatment resulted in 80.2% solid recovery, 97.5% cellulose recovery, 84.3% hemicellulose recovery

and 75.0% lignin removal, respectively. Comparisons of the above parameters in this work with those in other previous reports are shown in Table 3.

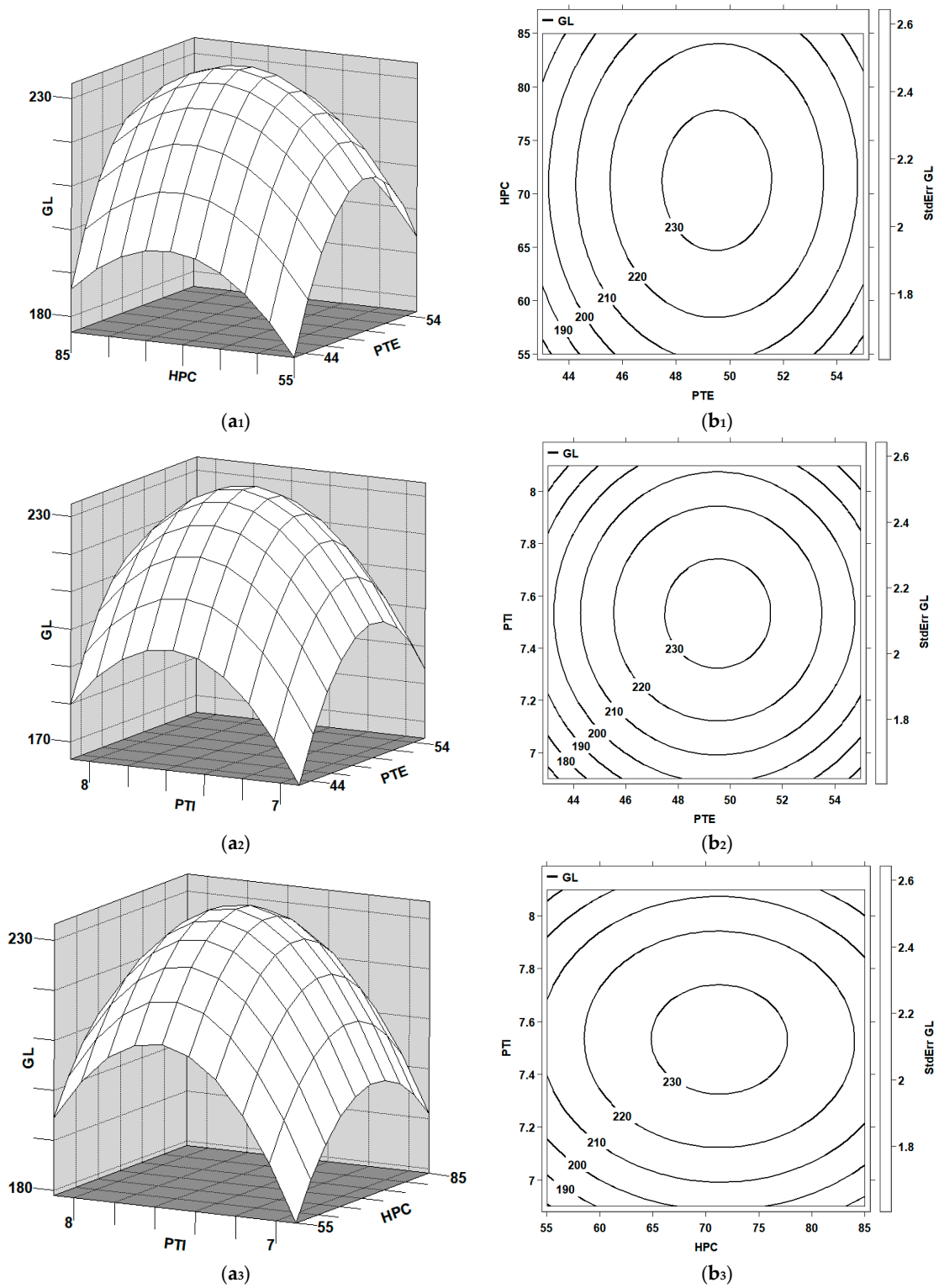


**Figure 1.** Response surface plots (**a**) and contour plots (**b**) of interaction effect of each independent variable's pair on glucose (GL, mg/$g_{ds}$). (**a1**,**b1**) PTE (pretreatment temperature, °C) and HPC (hydrogen peroxide concentration, g/L); (**a2**,**b2**) PTE (pretreatment temperature, °C) and PTI (pretreatment time, h); (**a3**,**b3**) HPC (hydrogen peroxide concentration, g/L) and PTI (pretreatment time, h).

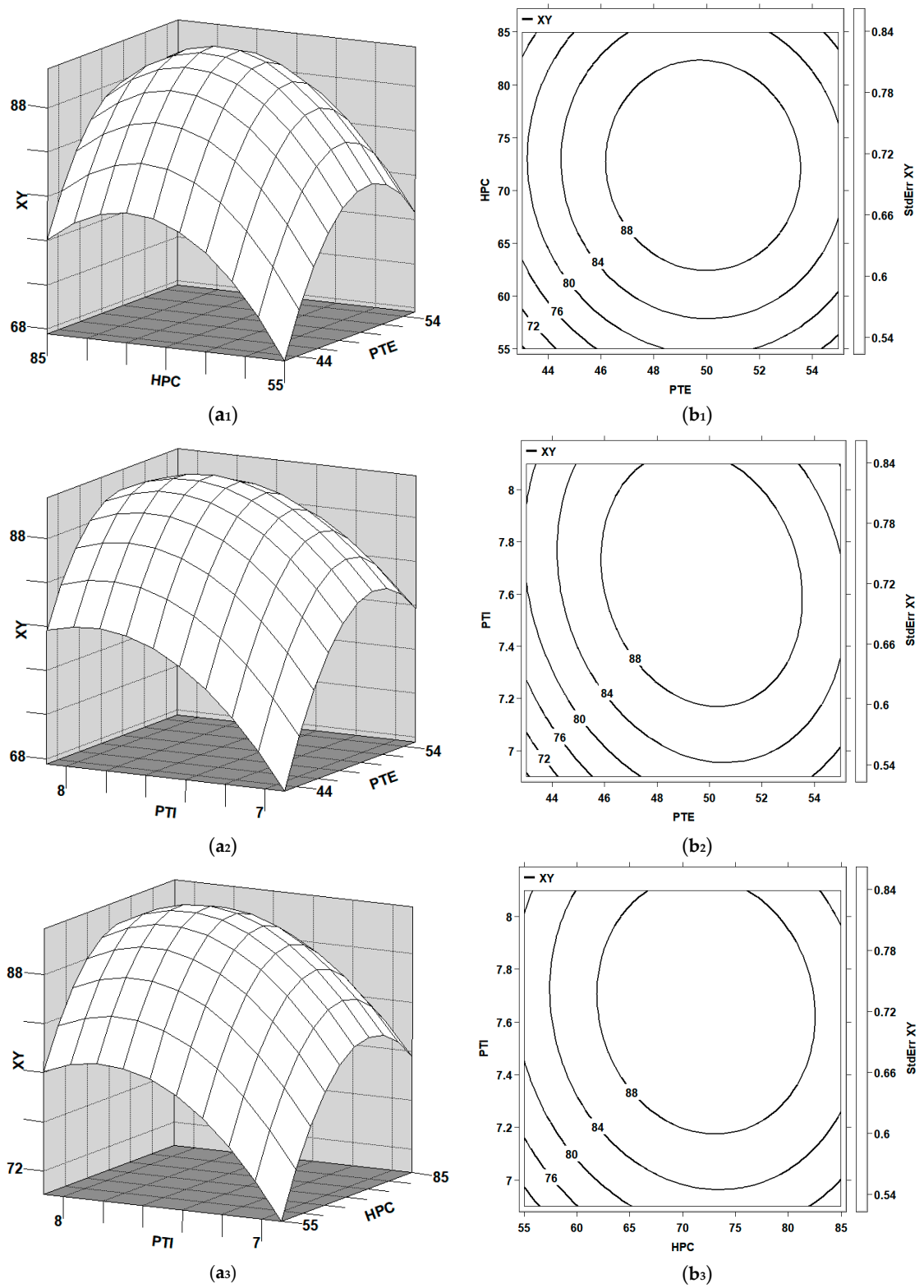


**Figure 2.** Response surface plots (**a**) and contour plots (**b**) of interaction effect of each independent variable's pair on xylose (XY, mg/$g_{ds}$). (**a**$_1$,**b**$_1$) PTE (pretreatment temperature, °C) and HPC (hydrogen peroxide concentration, g/L); (**a**$_2$,**b**$_2$) PTE (pretreatment temperature, °C) and PTI (pretreatment time, h); (**a**$_3$,**b**$_3$) HPC (hydrogen peroxide concentration, g/L) and PTI (pretreatment time, h).

**Table 3.** Comparisons of removal or recovery of main components in wheat straw, along with pretreatment conditions in different reports.

| Substrates | Pretreatment Conditions | CR (%) | HR (%) | LR (%) | References |
|---|---|---|---|---|---|
| Wheat straw | First step: alkaline hydrogen peroxide 3.0% (*w*/*v*), solid loading 40 g/L, 70 °C for 3.0 h; second step: lithium chloride/N, N-dimethylacetamide (LiCl/DMAc) solution (8.0%, *w*/*w*), solid loading 25 g/L, 110 °C for 2.5 h and room temperature for 12.0 h. | 92.59 | 31.39 | 95.2 | [23] |
| Wheat straw | First step: ammonium sulfite solution 20.0% (*w*/*w*), solid loading 167 g/L, 160 °C for 60 min; Second step: pretreatment using xylanase (66 U/$g_{ds}$), solid loading 50 g/L, 50 °C for 24.0 h. | 83.1 | 45.4 | 78.7 | [24] |
| Wheat straw | First step: toluene ethanol (2:1, *v*/*v*) for 6.0 h; second step: hot deionized water, solid loading 100 g/L, 200 °C for 0.5 h; third step: ethanol (70%, *v*/*v*) containing sodium hydroxide (1.0%, *w*/*v*), solid loading 50 g/L, 90 °C for 2.0 h. | 67.4 | 0 | 56.5 | [25] |
| Wheat straw | Sodium hydroxide solution 1.0% (*w*/*v*), solid loading 100 g/L, 30 °C for 6.0 h; mixture of sodium hydroxide solution (1.0%, *w*/*v*) and $H_2O_2$ (4.0 g/L), solid loading 100 g/L, 30 °C for 15.0 h. | 96.8 | 73.6 | 62.1 | [26] |
| Wheat straw | 50% cholinium alanine-glycerol, solid loading 50 g/L, 90 °C for 6.0 h. | 95.1 | 82.1 | 67.6 | [27] |
| Wheat straw | Mixture of choline chloride and monoethanolamine (1:6), solid loading 50 g/L, 70 °C for 9.0 h. | 93.7 | 57.9 | 71.4 | [28] |
| Wheat straw | Alkaline hydrogen peroxide solution 71 g/L, solid loading 200 g/L, 50 °C for 7.6 h. | 97.5 | 84.3 | 75.0 | This work |

CR: cellulose recovery; HR: hemicellulose recovery; LR: lignin removal.

It was obvious that cellulose recovery (97.5%) and hemicellulose recovery (84.3%) in this work were the most competitive among the different reports. Though the lignin removal of other studies, namely, 95.2% [23] and 78.7% [24], were higher than that (75.0%) in this work, the cellulose recovery (92.59%, 83.1%) and hemicellulose recovery (31.39%, 45.4%) in the two previous reports were lower than those (97.5%, 84.3%) in this work. This indicates that higher delignification could result in more degradation of holocellulose. Compared with the lignin removal in other studies, including 56.5% [25], 62.1% [26], 67.6% [27] and 71.4% [28], the lignin removal (75.0%) in this work was more competitive, which could facilitate the enzymatic digestibility of holocellulose. In conclusion, the optimized pretreatment conditions in this work could retain holocellulose in a large amount, along with simultaneously producing a relatively considerable lignin removal.

Relative to the pretreatment conditions in different reports in Table 3, the solid loading (200 g/L) adopted in this work was the most competitive, which could enhance pretreatment efficiency and reduce the requirement of reaction vessels. Of course, direct comparisons of temperature and time were not available, as higher temperature always corresponded with shorter time and lower temperature corresponded with longer time, respectively. Even so, the pretreatment temperature (50 °C) in this work was similar to that (50 °C) described by Yu et al. (2020) [24]. Meanwhile, the time (7.6 h) in this work was shorter than that (24 h) in the work described by Yu et al. (2020) [24], which indicates that the optimized pretreatment conditions in this work could enhance the pretreatment efficiency and reduce the energy cost for pretreatment. Although the time (7.6 h) in this work was longer than that (6.0 h) described by Zhao et al. (2017) [27], the higher solid loading (200 g/L) and lower temperature (50 °C) in this work were more competitive. Furthermore, compared with pretreatment conditions described by Li et al. (2019) [23], Yu et al. (2020) [24], Chen et al. (2018) [25] and Yuan et al. (2018) [26], the uncompli-

cated experimental operations in this work could reduce manual operations and facilitate large-scale application.

### *3.2. Optimization of Enzymatic Hydrolysis of the Pretreated Wheat Straw*

Tween-80 can protect the activation of cellulases, which may be decreased by lignin; swell the fiber; increase the surface area; and improve the adsorption of enzyme to cellulose [29]. Therefore, it was adopted during the enzymatic hydrolysis in this work. The statistical analysis of PBD in Table 4 indicated that the reaction time, enzyme loading and biomass loading had significant effects on glucose and xylose yields. Gupta and Parkhey (2014) [30] and Pandey and Negi (2015) [31] reported similar results for the enzymatic hydrolysis of fallen pine foliage and rice straw, respectively. The reaction time plays an important role during enzymatic hydrolysis. Insufficient reaction time can result in low sugar yields, whereas sugar yields always decrease in the later stage of enzymatic hydrolysis due to the recrystallization of cellulose and attachment of enzymes on the amorphous regions of cellulose. Enzyme loading always has an important effect on sugar yields. Too low of an enzyme loading is unfavorable to sugar yields, whereas too high of an enzyme loading is also adverse to sugar yields by increasing the rate of transglycosylation reactions, along with hydrodynamic instability, improper mixing and suspension of slurry [32]. Biomass loading also has an important role during enzymatic hydrolysis. Too low of a biomass loading can enhance the requirement of reaction vessels. Too high of a biomass loading can result in poor stirring, enzymatic feedback inhibition by end-products and a decrease in the synergistic action of cellulases. In this work, the Tween-80 concentration had an insignificant effect on the sugar yields, which was similar to the results reported by Jin et al. (2016) [33]. In addition, the reaction temperature had a negative effect and the reaction pH had a positive effect on sugar yields. Meanwhile, the reaction temperature had a significant on sugar yields during the enzymatic hydrolysis of sugarcane bagasse [34]. The reaction pH had a significant effect on the enzymatic hydrolysis of cotton stalk [35]. Different effects of the same factors on sugar yields were perhaps related to differences in substrates and cellulase properties. Based on the above results, enzymatic hydrolysis was carried out at 50 °C and pH 4.8 with a supplement of Tween-80 (0.3%, $w/v$) in the following steps.

**Table 4.** Coefficients of regression for glucose and xylose yields during optimization of enzymatic hydrolysis.

| Terms | $Y_3$ (Glucose, mg/$g_{ds}$) | $Y_4$ (Xylose, mg/$g_{ds}$) |
|---|---|---|
| Constant | 278.67 | 111.630 |
| A: Reaction time (h) | 25.63 $^{SS}$ | 9.403 $^{SS}$ |
| B: Reaction temperature (°C) | −1.15 | −1.582 |
| C: Enzyme loading (FPU/$g_{ds}$) | 16.47 $^{SS}$ | 8.090 $^{SS}$ |
| D: Reaction pH | 1.03 | 0.127 |
| E: Biomass loading (g/L) | 25.47 $^{SS}$ | 8.032 $^{SS}$ |
| F: Tween-80 concentration (%, $w/v$) | −0.72 | 1.278 |
| $R^2$ | 99.59% | 97.82% |
| Adj-$R^2$ | 99.18% | 95.64% |
| Lack of fit | 0.103 | 0.103 |

Outline criterion: 0.05; $^{SS}$ significant at 1% level.

The results of the steepest ascent method indicate that the glucose and xylose yields reached a plateau while the reaction time, enzyme loading and biomass loading were 36.0 h, 10.0 FPU/$g_{ds}$ and 90 g/L, respectively. The analysis results of the CCD are shown in Table 5. It indicates that the linear terms, namely, $X_1$, $X_2$ and $X_3$, and square terms, namely, ${X_1}^2$, ${X_2}^2$ and ${X_3}^2$, had significant effects on the glucose and xylose yields. The interaction terms, namely, $X_1X_2$, $X_1X_3$ and $X_2X_3$, had insignificant effects on the glucose yield. Meanwhile, only $X_1X_2$ had a significant effect on the xylose yield. High $F$ values of the models (199.12, 203.66) and lack of fit (3.40, 3.25), along with $p$-values of the models

($p$ = 0.000, $p$ = 0.000) and lack of fit ($p$ = 0.103, $p$ = 0.111), indicate that the obtained data were a good fit with the models. High values of $R^2$ (99.4%, 99.5%) and adjusted $R^2$ (98.9%, 99.0%) also demonstrated the accuracy of the models.

**Table 5.** Estimated regression coefficients for glucose and xylose yields during the optimization of enzymatic hydrolysis.

| Terms | Glucose ($Y_3$) | Xylose ($Y_4$) |
|---|---|---|
| Constant | 544.510 | 222.743 |
| Reaction time ($X_1$) | 15.597 $^{SS}$ | 6.926 $^{SS}$ |
| Enzyme loading ($X_2$) | 30.767 $^{SS}$ | 7.912 $^{SS}$ |
| Biomass loading ($X_3$) | −6.564 $^{SS}$ | −6.681 $^{SS}$ |
| Reaction time × reaction time ($X_1 \times X_1$) | −34.607 $^{SS}$ | −12.535 $^{SS}$ |
| Enzyme loading × enzyme loading ($X_2 \times X_2$) | −53.179 $^{SS}$ | −22.314 $^{SS}$ |
| Biomass loading × biomass loading ($X_3 \times X_3$) | −42.215 $^{SS}$ | −24.478 $^{SS}$ |
| Reaction time × enzyme loading ($X_1 \times X_2$) | 1.116 | 2.625 $^{S}$ |
| Reaction time × biomass loading ($X_1 \times X_3$) | 1.604 | 0.992 |
| Enzyme loading × biomass loading ($X_2 \times X_3$) | −2.759 | 1.045 |
| $R^2$ | 99.4% | 99.5% |
| Adj-$R^2$ | 98.9% | 99.0% |
| Lack of fit | 0.103 | 0.111 |

Outline criterion: 0.05; $^{S}$ significant at 5% level; $^{SS}$ significant at 1% level.

Three-dimensional response surface plots and the corresponding contour plots to demonstrate the combined effects of each variable's pair on glucose and xylose yields are shown in Figures 3 and 4, respectively. As shown in Figures 3($a_1$,$b_1$) and 4($a_1$,$b_1$), the optimal region of the reaction time was 35.0 h–37.5 h and that of the enzyme loading was 10.0 FPU/$g_{ds}$–12.0 FPU/$g_{ds}$ while the biomass loading was fixed at its middle level (90 g/L). As shown in Figures 3($a_2$,$b_2$) and 4($a_2$,$b_2$), the optimal region of the reaction time was 35.0 h–37.5 h and that of the biomass loading was 80 g/L–90 g/L while the enzyme loading was fixed at its middle level (10.0 FPU/$g_{ds}$). As shown in Figures 3($a_3$,$b_3$) and 4($a_3$,$b_3$), the optimal region of enzyme loading was 10.0 FPU/$g_{ds}$–12.0 FPU/$g_{ds}$ and that of biomass loading was 80 g/L–90 g/L while reaction time was fixed at its middle level (36.0 h).

According to canonical analysis, the maximal glucose yield (551.1 mg/$g_{ds}$) could be obtained while the pretreated WS was hydrolyzed at 50 °C and pH 4.8 for 36.9 h ($X_1$ = 0.22817) with 10.9 FPU/$g_{ds}$ of enzyme loading ($X_2$ = 0.29383) and 88 g/L of biomass loading ($X_3$ = −0.08301). The maximal xylose (224.9 mg/$g_{ds}$) could be obtained while the pretreated WS was hydrolyzed at 50 °C and pH 4.8 for 37.2 h ($X_1$ = 0.29130) by adopting 10.6 FPU/$g_{ds}$ of enzyme loading ($X_2$ = 0.19146) and 87 g/L of biomass loading ($X_3$ = −0.12648) with a supplement of Tween-80 (0.3% $w/v$).

After an adjustment, three confirmatory experiments for enzymatic hydrolysis were carried out at 50 °C and pH 4.8 for 37.0 h with the use of 10.8 FPU/$g_{ds}$ of enzyme loading and 88 g/L of biomass loading. The average yields of glucose (552.7 mg/$g_{ds}$) and xylose (223.6 mg/$g_{ds}$) could be obtained, which were close to the predicted values. Compared with the yields of glucose (234.2 mg/$g_{ds}$) and xylose (92.5 mg/$g_{ds}$) before optimization, the optimization resulted in a 1.36-fold increase in the glucose yield and a 1.42-fold increase in the xylose yield. After the calculation, cellulose and hemicellulose conversion were 94.0% and 83.5%, respectively.

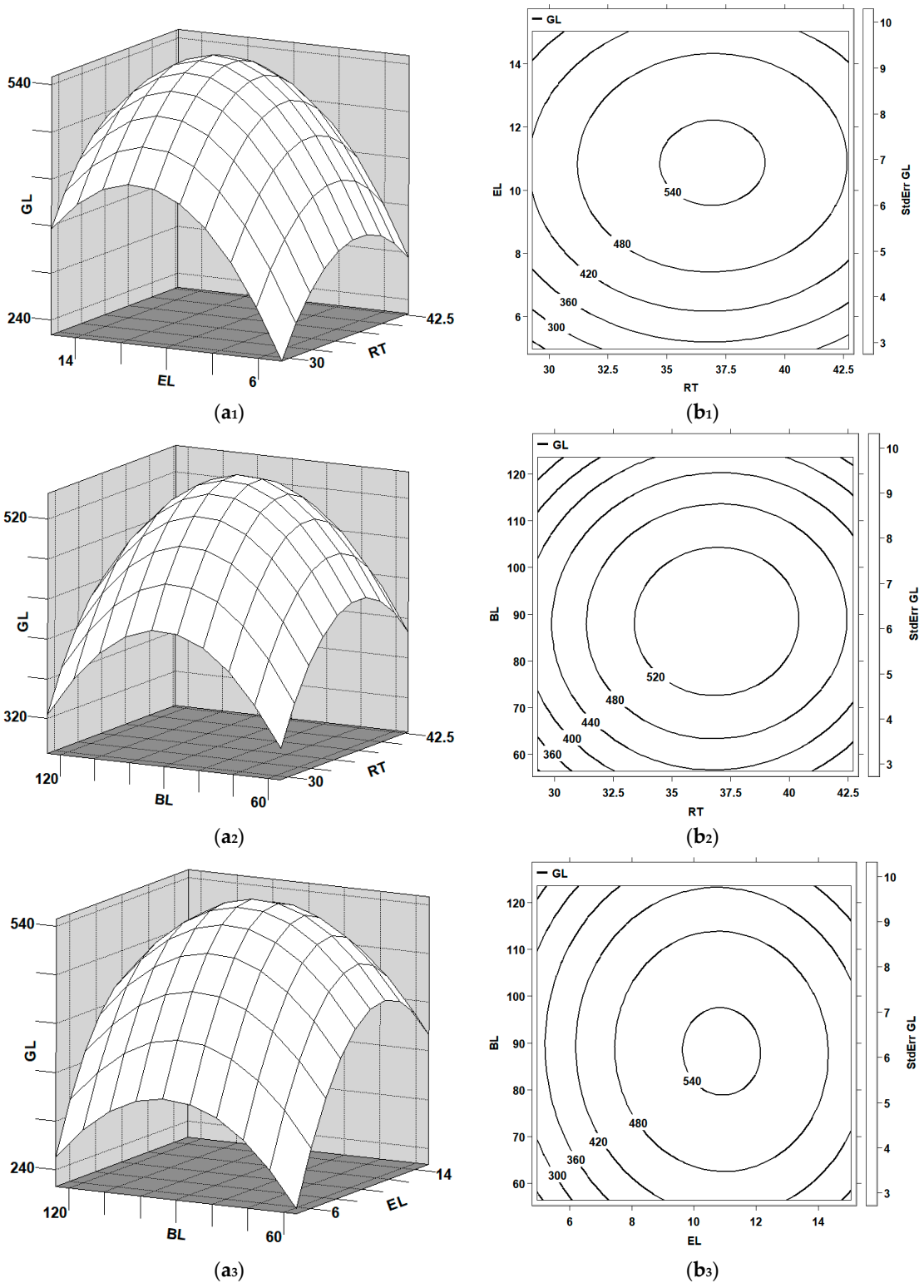


**Figure 3.** Response surface plots (**a**) and contour plots (**b**) of interaction effect of each independent variable's pair on glucose (GL, mg/$g_{ds}$). (**a**$_1$,**b**$_1$) RT (reaction time, h) and EL (enzyme loading, FPU/$g_{ds}$); (**a**$_2$,**b**$_2$) RT (reaction time, h) and BL (biomass loading, g/L); (**a**$_3$,**b**$_3$) EL (enzyme loading, FPU/$g_{ds}$) and BL (biomass loading, g/L).

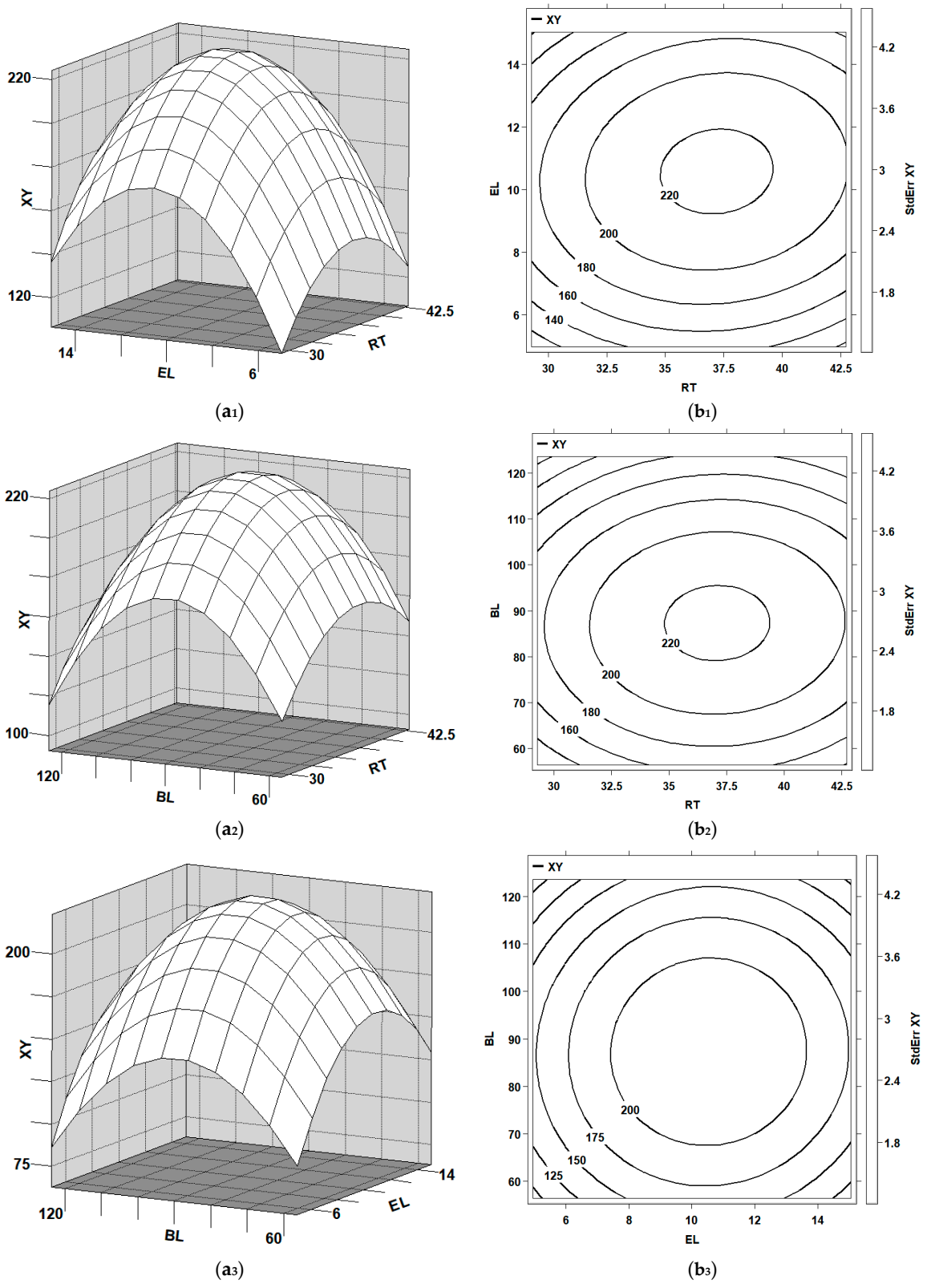


**Figure 4.** Response surface plots (**a**) and contour plots (**b**) of interaction effect of each independent variable's pair on xylose (XY, mg/$g_{ds}$). (**a**$_1$,**b**$_1$) RT (reaction time, h) and EL (enzyme loading, FPU/$g_{ds}$); (**a**$_2$,**b**$_2$) RT (reaction time, h) and BL (biomass loading, g/L); (**a**$_3$,**b**$_3$) EL (enzyme loading, FPU/$g_{ds}$) and BL (biomass loading, g/L).

Comparisons of sugar yields, along with enzymatic hydrolysis conditions, between different reports are shown in Table 6. It is obvious that the glucose yield (562.9 mg/$g_{ds}$) described by Yu et al. (2020) [24] was higher than that (552.7 mg/$g_{ds}$) in this work. Compared with the other six reports, the glucose yield (552.7 mg/$g_{ds}$) in this work was more competitive. As shown in Table 6, the xylose yield (223.6 mg/$g_{ds}$) in this work was higher than those in the other five reports. Though the higher glucose yield (562.9 mg/$g_{ds}$) and shorter reaction time (12 h) described by Yu et al. (2020) [24] were more competitive than those in this work, the adoption of commercial cellulases and lower biomass loading (30 g/L) could increase the enzyme cost and requirement of reaction vessels. Compared with the biomass loading and reaction time in the other six reports, the higher biomass loading (88 g/L) and shorter reaction time (37.0 h) in this work could reduce the enzymatic hydrolysis cost and enhance hydrolysis efficiency. Direct comparisons of enzyme loading in different reports were not available, as the enzyme assay conditions differed from each other. Even so, the enzyme loading (10.8 FPU/$g_{ds}$) in this work was lower than 66.3 FPU/$g_{ds}$ [24], 38.2 FPU/$g_{ds}$ [27,28], 40.4 FPU/$g_{ds}$ [36], 39.7 FPU/$g_{ds}$ [37] and 53.9 FPU/$g_{ds}$ [38]. The adoption of lower enzyme loading could reduce the enzyme input cost. Though 10.0 FPU/$g_{ds}$ adopted by Patel et al. (2017) [39] was lower than that (10.8 FPU/$g_{ds}$) in this work, the adoption of commercial cellulases in that work could enhance the enzyme input cost. In addition, the cellulose and hemicellulose conversions (94.0%, 83.5%) in this work were the most competitive among the reports in Table 6. Higher cellulose conversion and hemicellulose conversion were related to higher enzymatic digestibility of holocellulose in the pretreated WS. Therefore, the optimized enzymatic hydrolysis conditions could enhance the sugar yields, along with higher cellulose and hemicellulose conversion, by using a lower enzyme loading, higher biomass loading and shorter reaction time.

**Table 6.** Comparisons of enzymatic hydrolysis conditions of wheat straw along with glucose and xylose yields in different reports.

| Substrates | Enzyme Sources | Enzymatic Hydrolysis Conditions | Glucose (mg/$g_{ds}$) | Xylose (mg/$g_{ds}$) | Cellulose Conversion (%) | Hemicellulose Conversion (%) | References |
|---|---|---|---|---|---|---|---|
| Wheat straw | Commercial cellulases from Qingdao Vland Biotech Inc. Qingdao, China. | Enzyme loading 66.3 FPU/$g_{ds}$, biomass loading 30 g/L, 12 h. | 562.9 | – | 90.3 | – | [24] |
| 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 | [27] |
| Wheat straw | Cellulast 1.5 L® from Novozymes | Enzyme loading 38.2 FPU/$g_{ds}$, biomass loading 10 g/L, 72 h. | 478.93 | 131.05 | 89.8 | 62.0 | [28] |
| Wheat straw | Cellic® CTec2 from Novozymes | Enzyme loading 40.4 FPU/$g_{ds}$, biomass loading 50 g/L, 72 h. | 425.2 | – | 77.3 | – | [36] |
| Wheat straw | Cellic® CTec2 from Novozymes | Enzyme loading 39.7 FPU/$g_{ds}$, biomass loading 50 g/L, 72 h. | 505.12 | ≈0 | 92.9 | ≈ 0 | [37] |
| 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 | [38] |
| 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 | [39] |
| Wheat straw | *Aspergillus niger* HQ-1 | Enzyme loading 10.8 FPU/$g_{ds}$, biomass loading 88 g/L, 37.0 h. | 552.7 | 223.6 | 94.0 | 83.5 | This work |

## 4. Conclusions

The adoption of high solid loading (200 g/L) under the optimized pretreatment conditions and biomass loading (88 g/L) under the optimized enzymatic hydrolysis conditions could reduce the vessel and input costs for sugar production. Satisfactory levels of cellulose

recovery (97.5%), hemicellulose recovery (84.3%) and lignin removal (75.0%) could be obtained under the optimized pretreatment conditions. Considerable glucose (552.7 mg/$g_{ds}$) and xylose (223.6 mg/$g_{ds}$) yields, along with higher cellulose conversion (94.0%) and hemicellulose conversion (83.5%), could be obtained under the optimized enzymatic hydrolysis conditions. In addition, the adoption of self-produced cellulases for enzymatic hydrolysis in this work could also reduce the enzyme cost for sugar production. In the future, the optimization of bioethanol production utilizing fermentable sugar from the pretreated WS will be carried out to investigate the application prospects of the results found in this work.

**Supplementary Materials:** The following supporting information can be downloaded from https://www.mdpi.com/article/10.3390/fermentation9100871/s1, Supplementary materials S1: Materials and methods for optimization of cellulases extraction from *Aspergillus niger* HQ-1; Supplementary materials S2: Results of optimization of cellulases extraction from *Aspergillus niger* HQ-1; Table S1: Codes and levels of four variables and Plackett-Burman design (PBD) along with filter paper activity during optimization of cellulases extraction; Table S2: Coefficients of regression for filter paper activity by *Aspergillus niger* HQ-1; Table S3: Design of the steepest ascent method along with filter paper activity during optimization of cellulases extraction; Table S4: Codes and levels of variables and BBD along with filter paper activity during optimization of cellulases extraction; Table S5: Estimated regression coefficients for filter paper activity during optimization of cellulases extraction; Table S6: Analysis of variance (ANOVA) for the fitted quadratic polynomial model for cellulases extraction; Table S7: Codes and levels of five variables and Plackett-Burman design (PBD) along with glucose and xylose yields during optimization of pretreatment; Table S8: Design of the steepest ascent method along with glucose and xylose yields during optimization of pretreatment; Table S9: Codes and levels of variables and BBD along with glucose and xylose yields during optimization of pretreatment; Table S10: Codes and levels of six variables and Plackett-Burman design (PBD) along with glucose and xylose yields during optimization of enzymatic hydrolysis; Table S11: Design of the steepest ascent method along with glucose and xylose yields during optimization of enzymatic hydrolysis; Table S12. Codes and levels of variables and CCD along with glucose and xylose yields during optimization of enzymatic hydrolysis.

**Author Contributions:** H.Z. conceived and designed all the experiments. H.Z. performed the pretreatment optimization experiments and analyzed the data. J.W. performed the experiments including optimization of enzymatic hydrolysis and cellulases extraction by *A. niger* HQ-1 and analyzed the data. H.Z. wrote, reviewed and edited the manuscript. All authors have read and agreed to the published version of the manuscript.

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## Abbreviations

| | |
|---|---|
| AHP | Alkaline hydrogen peroxide |
| WS | Wheat straw |
| RSM | Response surface methodology |
| PBD | Plackett–Burman design |
| CCD | Central composite design |
| BBD | Box–Behnken design |
| FPU/mL | Filter paper activity unit per milliliter |
| XU/mL | Xylanase activity unit per milliliter |
| FPU/$g_{ds}$ | Filter paper activity unit per gram dry substrate |
| mg/$g_{ds}$ | Milligram per gram dry substrate |
| SR | Solid recovery |
| CR | Cellulose recovery |
| HR | Hemicellulose recovery |
| LR | Lignin removal |
| PTE | Pretreatment temperature |
| HPC | Hydrogen peroxide concentration |
| PTI | Pretreatment time |
| GL | Glucose |
| XY | Xylose |
| RT | Reaction time |
| EL | Enzyme loading |
| BL | Biomass loading |

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