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Liquid hot water pretreatment combined with high-solids enzymatic hydrolysis and fed-batch fermentation for succinic acid sustainable processed from sugarcane bagasse

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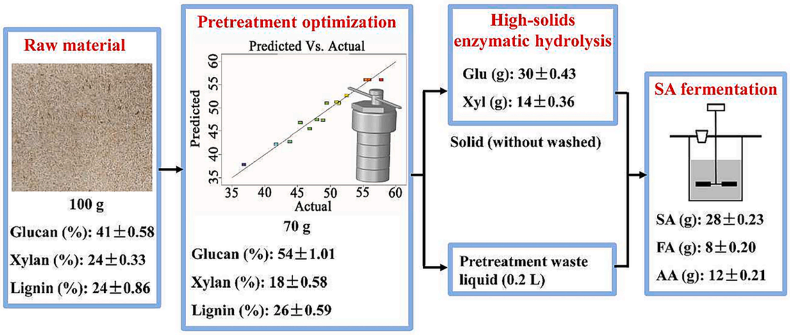
HIGHLIGHTS

- Realized efficient high-solids enzymatic hydrolysis of unwashed sugarcane bagasse.
- Achieved pretreatment liquid emission reduction and C5/C6 sugars full utilization.
- Pretreatment, enzymatic hydrolysis and fermentation procedures were coupled and optimized.
- Established sustainable process for bio-refining succinic acid from sugarcane bagasse.

GRAPHICAL ABSTRACT



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ABSTRACT

In order to sustainable process of bio-succinic acid (SA), response surface methodology (RSM) was applied to optimize liquid hot water pretreatment pretreatment of sugarcane bagasse (SCB), followed by high-solids enzymatic hydrolysis of pretreated residual that without washing, then the hydrolysates and partial pretreatment liquid were used as carbon sources for SA fermentation. Results showed that the highest sugars yield could be achieved at pretreatment conditions of temperature 186 °C, time 25 min and solid-to-liquid ratio 0.08; enzymatic digestion the pretreated residuals at 20 % (w/v) solid content via enzymes reconstruction and fed-batch strategy, the obtained sugars reached to 121 g/L; by controlling the nutrition and conditions of the fermentation process, most of the C5 and C6 sugars in the hydrolysate and pretreatment liquid were converted into SA with a conversion rate high to 280 mg/g SCB. This study can provide a novel clue for clean and efficient biorefining of chemicals.

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1. Introduction

Green plants convert carbon dioxide and water into carbohydrates by absorbing light energy is a green and sustainable process, the annual output of lignocellulose in the world high to billions of tons, the application of biorefinery technology to convert it into high-value chemicals and fuels, etc., is expected to reduce the use of fossil and alleviate the contradiction between resources, environment and development (Lu et al., 2021). Compared with chemical synthesis methods, biorefinery approaches generally have the characteristics of clean processing, low waste gas/liquid emissions and sustainability. However, the dispersedly distributed lignocellulosic biomass lead to high cost of substrate collection and transportation, therefore, whether it is easy to be collected is one of the key factors to determine the economic benefits of biorefinery (Singhania et al., 2022). Cane sugar is one of the most important edible sugars for human beings, it is mainly processed from sugarcane, during the processing of cane sugar, a large amount of by-product sugarcane bagasse would be produced, and its carbohydrates content high to 45–65 % (w/w) and easy to be collected, is recognized as an ideal feedstock for biorefinery (Ajala et al., 2021). In recent years, SCB was widely used as feedstock for substitution of unrenewable fossil to produce a variety of chemicals, including organic acids and liquid fuels, etc (Mustafa et al., 2020).

Biorefinery aims to convert carbohydrates in lignocellulosic biomass into high-value products via microbial fermentation, therefore, efficient release of fermentable sugars from lignocellulose is a key step (Houfani et al., 2020). The main chemical components of lignocellulosic biomass including cellulose, hemicellulose and lignin, wherein lignin and hemicellulose are filled in fiber bundles by covalent bonding to form a dense three-dimensional network structure that is highly resistant to degradation (Singhania et al., 2022). Destruction of the anti-degradation structure of lignocellulosic biomass helps promote the release of fermentable sugars, commonly used approaches including chemical, physical and biological pretreatment, etc. Among different pretreatment approaches, chemical pretreatment generally exhibits high efficiency but causes environmental pollution, physical pretreatment is chemicals free but demand for high energy input, biological pretreatment has green process but with low efficiency (Prasad et al., 2022). Liquid hot water pretreatment is generally performed between 160 °C and 250 °C with a reaction pressure higher than its saturated vapor pressure, the properties of water would change with the increase of pretreatment temperature and ionize a large amount of H^+ and OH^-, therefore exhibiting the characteristics of acid catalysis and alkali catalysis (Serna-Loaiza et al., 2022). Meanwhile, the destruction of the hydrogen bond network structure at high temperature enhances the diffusivity of water molecules, and the diffusion coefficient of supercritical water molecules is 10 to 100 times higher than that of ordinary water, which makes its movement speed and mass transfer rate in the separation process are greatly increased, so it has better fluidity, permeability and transfer performance, and favor to mass transfer and heat exchange (Chen et al., 2021). Liquid hot water pretreatment does not require chemicals and hardly formation of inhibitors, has aroused increasing attention in recent years (Jimenez-Gutierrez et al., 2021; Phuttaro et al., 2019).

The release of fermentable sugars in lignocellulose via enzymatic hydrolysis process is one of the key steps of biorefinery. Generally, obtaining a high concentration of fermentable sugars is conducive to adapting subsequent diversified fermentation strategies, producing high-concentration fermentation products, and reducing the cost of downstream refining (da Silva et al., 2020; Houfani et al., 2020). Therefore, enzymatic hydrolysis with high substrate dosage (solid content > 15 % (w/v)) is an inevitable choice for biorefinery. However, due to the lack of free water in the high-solids enzymatic hydrolysis system, resulting in high viscosity and poor mobility, which would hinder the saccharification of lignocellulosic feedstocks (da Silva et al., 2020). In addition, the high cost of cellulase is also one of the bottleneck problems restricting the large-scale application of lignocellulosic biomass biorefinery technology for a long time (Houfani et al., 2020). In the process of enzymatic hydrolysis, the proportional imbalance of different cellulolytic enzymes and the non-productive adsorption of enzymes by lignin would lead to the inefficient saccharification of lignocellulosic feedstocks, some additives and accessory enzymes were proved to enhance enzymatic hydrolysis efficiency (Xu et al., 2019a). In order to alleviate the adverse effects such as lack of free water, low heat and mass transfer efficiency caused by high substrate content during high-solids enzymatic hydrolysis process, the strategy of adding substrates in batches to maintain the actual solids content at a low level, thereby improving the fluid performance and boosting the enzymatic saccharification of lignocellulosic feedstocks (Battista and Bolzonella, 2018).

SA exhibit excellent reactivity ascribed to it possess two functional carboxyl groups, which can be used as precursor in the synthesis of various valuable chemicals such as alcohols, esters and degradable polymer compounds, etc (Lu et al., 2021; Zhang et al., 2022). Due to the SA has important application value in food, medicine and other fields, its current annual global market high to 200,000 tons, and the demand is rising year by year (Xu et al., 2018; Zhang et al., 2022). SA is mainly synthesized through chemical approaches such as paraffin oxidation and maleic anhydride hydrogenation, however, these technologies require the use of unsustainable petroleum as substrate and causes serious environmental pollution (Ladakis et al., 2022). The application of biorefinery technology for SA processed from lignocellulosic feedstocks can not only overcome the disadvantages of unsustainable raw materials and process pollution in traditional processes, but also with the potential of reducing the production cost by 80 % (Dickson et al., 2021; Lu et al., 2021). The popular strains for SA fermentation mainly include *Actinobacillus succinogenes*, *Yarrowia lipolytica*, *Saccharomyces cerevisiae*, *Anaerobiospirillum succiniciproducens*, *Basfia succiniciproducens* and *Escherichia coli*, etc. Among them, *A. succinogenes* with the capability of utilizing many kinds of carbon sources such as glucose, xylose, cellobiose, sorbitol, etc. to produce SA, which is considered as the most promising natural SA-producing strain for industrial application (Xu et al., 2021a; Yang et al., 2020). Lee et al. (2022) tried to process SA from Napier grass and glycerol, the obtained SA yield reached to 0.88 g/g substrate after optimization the ratio of different carbon sources. There are differences in the capability of *A. succinogenes* to convert different lignocellulosic feedstocks to produce SA, for example, when using the hydrolysates of olive pits, SCB and pure xylose as carbon sources for fed-batch SA fermentation, the achieved SA concentrations were 36.7 g/L, 33.6 g/L and 28.7 g/L, respectively (Jokodola et al., 2022).

In present study, the optimization of liquid hot water pretreatment conditions was carried out via response surface methodology; subsequently, the without washed pretreated residue was suffered to high-solids enzymatic hydrolysis with the help of compound enzymes and fed-batch strategies; after that, the fermentable sugars released during pretreatment and enzymatic hydrolysis were converted into SA using a fed-batch fermentation strategy. Through overall optimization and improvement of biorefinery technology, this study has established a clean and sustainable approach for SA processed from sugarcane bagasse, which provides new clues for the preparation of high valuable bio-based chemicals from lignocellulosic feedstocks.

2. Materials and methods

2.1. Raw materials and chemicals

SCB raw material was collected from the cane sugar factory in Nanning, China, it was crushed to 20 – 60 mesh and dried to constant weight. The main components of SCB (based on dried substrate) were measured as: cellulose 41 %, hemicellulose 24 %, lignin 24 %, moisture 1.7 %, ash 2.3 % and extract contents 2.6 %. The chemicals reagents were supplied by Macklin Biochemical Co., Ltd (Shanghai, China), the Cellic® CTec3 (the activities of cellulase and hemicellulase were 150 FPU/mL and 1800 U/mL with total protein concentration of 273 mg/

mL) and HTec3 (9000 U/mL) were provided by Novozymes A/S (Bagsværd, Denmark). Sophorolipid was purchased from Pioneer Biotech Co., Ltd (Shanxi, China) (purity, 99 %; MW, 688.8 Da; pH value was 4.0 – 6.0; the density and acidity coefficient (pKa) were 1.152 ± 0.06 g/cm^3 and 1.152 ± 0.06 g/cm^3, respectively) (Xu et al., 2021b).

2.2. Pretreatment methods

Liquid hot water pretreatment of SCB were performed in a 100 mL cylindrical pressure resistant reactor with working volume of 60 mL, the heating procedure was: first heat to 100 °C for 15 min, then heat to the set temperature for a specific time, after the reaction, the reactor was cooled with cold water bath. The experiments were carried out according to the three factor Box Behnken design that response to sugars yield, in specific, the conditions of reaction time, temperature and solid-to-liquid (S: L) ratio were 10–40 min, 160–210 °C and 0.04–0.12, respectively (illustrated in Table 1). After pretreatment, the solid and liquid fraction was separated via vacuum filtration, the solid residue was washed to natural pH and dried to constant weight (the substrates used except for response surface optimization were without washed and dried) prior to usage, the pretreatment liquid was collected to detect pH value and measure the content of sugars, phenols and acids. The seeds were prepared by cultivation.

2.3. Enzymatic hydrolysis

Batch enzymatic hydrolysis were carried out at 150 rpm and 50 °C in a 100 mL glass bottle with rubber stopper (the working volume was 40 mL 0.2 M sodium acetate buffer with pH value of 4.8). The effects of feedstocks (unwashed and undried) addition amount of 5 %, 10 %, 15 %, 20 % (w/v), cellulase loading of 10, 20, 30, 40 FPU/g dry material (DM), hemicellulase dosage of 0, 200, 400, 600, 800 U/g DM, sophorolipids loading of 0, 20, 40, 60, 80, 100 mg/g DM on the efficiency of enzymatic hydrolysis were evaluated. High-solids (20 %, w/v) enzymatic hydrolysis of pretreated feedstocks (unwashed and undried) was carried out in a 500 mL glass bottle with rubber stopper (with working volume of 300 mL) according to the below fed-batch strategies: all enzymes and additives were added into the reaction system at initial stage, then addition of 2–8 % (w/v) at 0, 6, 12 and 18 h, after digestion for specific time, 0.5 mL mixture was sampled for sugars measurement.

2.4. SA fermentation

The seeds were prepared by cultivation of *A. succinogenes* ATCC 55,618 in Tryptic soy broth (TSB) (Sigma-Aldrich, USA) at 37 °C for 18 h, then transferred the seeds into fermentation medium (L) (0.3 g Na_2HPO_4, 0.5 g $CaCl_2$, 1.0 g NaCl, 1.4 g NaH_2PO_4, 0.5 g $MgCl_2$, 1.5 g K_2HPO_4, 10 g yeast extract and appropriate addition amount of SCB hydrolysate) to perform SA fermentation, the volume of fermenter and working volume were 1 L and 600 mL, during this process, the broth was flushed with 0.5 mL/min CO_2 and the pH value was maintained at 6.8 by pumping 5 M NaOH, respectively.

2.5. Structural characterization

Scanning electron microscopy (acceleration voltage set at 2.0 kV) was applied to observe the surface morphology of different SCB samples. The FTIR spectra (from 4000 to 800 cm^{-1}) of various samples were characterized by Fourier transform infrared spectroscopy (Bruker, Germany). The crystal types of different samples were analyzed with the help of X-ray diffractometer (Philips, Holland).

2.6. Analytical methods

The main chemical components of sugarcane bagasse were analyzed according to the description of National Renewable Energy Laboratory (NREL) (Sluiter et al., 2012). The quantitative determination of glucose, SA, formic acid (FA), xylose and acetic acid (AA) were conducted following previous studies (Xu et al., 2021a; Zhang et al., 2022).

2.7. Statistical analysis

All the data presented in this study (mean ± SD) were based at least triplicate experiments, and data analysis was carried out with the help of IBM SPSS Statistic 19 software.

$$\text{Sugars yield}(\%) = (\text{glucose} + \text{xylose})(\text{g/L}) \times \text{volume}(\text{L}) \times 100/\text{theoretical mass of glucose and xylose in SCB raw material mass}(\text{g}) \tag{1}$$

(glucose + xylose), the concentrations of glucose and xylose in enzymatic hydrolysate; volume, volume of enzymatic hydrolysis system.

$$\begin{aligned}\text{SA yield}(\%) = {} & \text{SA concentration}(\text{g/L}) \times \text{volume}(\text{L}) \\ & \times 100/\text{consumed concentration of glucose and xylose}(\text{g/L}) \\ & \times \text{volume}(\text{L})\end{aligned} \tag{2}$$

SA concentration, concentration difference of SA before and after fermentation; volume, volume of fermentation system; consumed concentration of glucose and xylose, concentration difference of glucose and xylose before and after fermentation.

$$\text{SA conversion rate}(\%) = \text{SA mass}(\text{g}) \times 100/\text{consumed SCB mass}(\text{g}) \tag{3}$$

SA mass, SA produced by fermentation of sugarcane bagasse hydrolysate as carbon source; consumed SCB mass, the mass of sugarcane bagasse raw materials required for preparing the carbon source in the consumed hydrolysate.

$$\text{Glucan/Xylan recovery rate}(\%) = \text{glucan/xylan mass in pretreated residual}(\text{g}) \times 100/\text{glucan/xylan mass in SCB raw material}(\text{g}) \tag{4}$$

$$\text{CrI} = (\text{I}_{\text{main}} - \text{I}_{\text{am}})/\text{I}_{\text{main}} \times 100\% \tag{5}$$

I_{main}, the peak near to $2\theta = 22.5^\circ$ (cellulose I) / or $2\theta = 20.1^\circ$ (cellulose II); I_{am}, the valley between the main and secondary peaks (Xu et al., 2019b).

$$\log R_0 = \log(t \times \exp((T - 100)/14.75)) \tag{6}$$

$\log R_0$, severity factor (SF); T, temperature (°C); t, reaction time (min) (Kang et al., 2020).

Table 1
Three-factorial Box-Behnken design.

Variable	Symbol	Coding level		
		−1	0	1
Temperature, °C	A	160	185	210
Time, min	B	10	25	40
Solid-to-liquid ratio (w/v)	C	0.04	0.08	0.12

3. Results and discussion

3.1. Pretreatment of SCB at different conditions

Although applying liquid hot water pretreatment of lignocellulosic feedstocks to boost its degradability has the advantages of chemicals free and high efficiency, this process often requires high energy consumption and causes C-5 sugar loss. In order to enhance sugar conversion efficiency and reduce energy input, the optimization of pretreatment conditions was carried out. The effects of reaction temperature, time and S:L on LHW pretreatment of SCB were assessed (Table 2). During hydrothermal pretreatment process, changing temperature, time and S:L had limited effect on lignin removal rate. Increasing the temperature aggravates the acidification of the reaction system and leading to more hemicellulose dissolved, however, cellulose also would be hydrolyzed when the temperature was too high. Moreover, high temperature may also lead to further degradation of lignocellulosic biomass to form derivatives that inhibiting enzymatic hydrolysis and/ or microbial fermentation (Kang et al., 2020). Similarly, prolonging the pretreatment time would result in the loss of carbohydrate, so pretreatment time is another critical factor that affecting sugars yield. In general, performing pretreatment at high solid-to-liquid ratio help to increase efficiency of equipment, but it will lead to high viscosity, which is not conducive to the heat and mass transfer of the system. Present study found that changing S: L (0.04–0.12) had limited effects on different chemical compositions of SCB.

In general, liquid hot water pretreatment is performed at high pressures and high temperatures (ranged from 160 to 240 °C), under this condition, the acidic hydronium (H_3O^+) ions derived from water would result in an acid reaction environment, the ionized water (H_3O^+, OH^- plasma) is considered to be more efficient to break chemical bonds within/between lignin, cellulose and hemicellulose more efficiently than acetic or formic acid (Barbier et al., 2012). Furthermore, as the temperature increased from 25 °C to 220 °C, the ionic constant of water increases from 10^{-14} to 10^{-11}, and its reactivity would obviously increase as the disproportionation of water (Peterson et al., 2008). However, the ionic constant of water would rise rapidly as the temperature increase to over 200 °C, resulting in the large hydrolysis of cellulose and formation of organic acids and aldehydes, which would cause potential inhibitory effect on enzymatic hydrolysis and microbial fermentation (Chen et al., 2021; Xu et al., 2020). Therefore, to minimize the loss of cellulose and formation of inhibitors during pretreatment, another strategy is prolonging pretreatment time at relative low temperature (Sun et al., 2022).

Optimum conditions for pretreatment of different lignocellulosic biomass feedstocks with liquid hot water pretreatment are often various, in order to compare the intensity of different pretreatment approaches, the severity factor (SF, logR0) was introduced (Batista et al., 2019). The relationships between SF and pH value, solid/ cellulose/ hemicellulose recovery rate, lignin removal rate are illustrated in Fig. 1. The SF exhibited an obvious negative correlation ($R^2 > 0.9$) with the pH value of the reaction system, which can be attributed to the increased ionization of water due to the rise in pretreatment intensity, resulting in the production of a large amount of H_3O^+ (showed in Fig. 1 (a)) (Batista et al., 2019). As the results presented in Fig. 1(b)–(d), the solid/ glucan recovery rate and hemicellulose recovery rate also exhibited negative correlation with the SF, while rising SF within a certain range ($2.7 < SF < 4.9$) showed no significant effect on lignin removal rate (Fig. 1(e)).

It is well known that hemicellulose, as the main component in lignocellulosic biomass, acting as a physical barrier during enzymatic hydrolysis process. Liquid hot water pretreatment of lignocellulosic biomass can contribute to remove hemicellulose, the degradability of feedstocks was obviously enhanced as the SF increased, within the range of from 2.76 to 4.94, increasing SF will favor to achieve higher glucose yield (Fig. 1(f)). Although increasing SF help to boost the enzymatic hydrolysis of hemicellulose (Fig. 1(g)), it will cause hemicellulose loss in the pretreatment process, leading to a decrease in total sugar yield (Fig. 1(h)). This could be attributed to the partial removal of hemicellulose help to increase the specific surface area of the substrate, thereby enhancing the accessibility of enzymes to the substrate (Chen et al., 2021; Yu et al., 2016). Since the liquid hot water pretreatment process hardly removes lignin, it is necessary to evaluate the effect of lignin in the residue on enzymatic hydrolysis, the previous study has proved that the lignin in liquid hot water pretreated SCB exhibited limited inhibitory effect on enzymatic digestion (Xu et al., 2020). Therefore, the key to achieve the highest possible sugar yield is to control the severity factors of liquid hot pretreatment so as to maintain a balance between hemicellulose loss and increase the total sugars yield (Kang et al., 2020). Quintero et al. (2021) found that the hemicellulose removal rate had obvious positive correlation with digestibility of lignocellulosic feedstocks (especially when the solid content > 15 % (w/v)).

Table 2
Chemical component and response value of sugarcane bagasse pretreatment with liquid hot water.

No.	Tem. (°C)	T (min)	S: L	SF	pH	SRR (%)	Glu (%)	Xyl (%)	Lin (%)	SY (%)
1	210	10	0.08	4.24	4.3 ± 0.06	59 ± 0.80	63 ± 1.00	2 ± 0.23	31 ± 1.23	52 ± 1.27
2	160	50	0.08	3.47	4.9 ± 0.07	73 ± 0.80	52 ± 0.58	19 ± 0.58	25 ± 1.18	51 ± 0.22
3	185	50	0.12	4.20	4.2 ± 0.13	57 ± 1.60	64 ± 0.27	3 ± 0.17	32 ± 0.75	53 ± 0.12
4	210	50	0.08	4.94	3.2 ± 0.11	48 ± 2.02	64 ± 0.99	0 ± 0.00	35 ± 0.89	44 ± 1.31
5	160	30	0.04	3.24	5.1 ± 0.08	75 ± 0.66	50 ± 0.55	23 ± 0.91	24 ± 0.52	46 ± 1.42
6	210	30	0.04	4.72	3.6 ± 0.04	53 ± 0.57	65 ± 1.00	0 ± 0.00	35 ± 0.63	47 ± 0.27
7	185	30	0.08	3.98	4.6 ± 0.07	70 ± 0.56	51 ± 0.45	15 ± 0.63	26 ± 0.70	56 ± 0.94
8	185	10	0.04	3.50	4.8 ± 0.10	72 ± 1.24	53 ± 0.54	20 ± 0.73	25 ± 1.01	52 ± 1.34
9	185	30	0.08	3.98	4.8 ± 0.07	69 ± 0.59	53 ± 0.70	18 ± 0.45	27 ± 0.43	55 ± 0.79
10	185	30	0.08	3.98	4.6 ± 0.05	70 ± 1.17	51 ± 0.56	17 ± 0.59	26 ± 0.46	55 ± 0.51
11	185	30	0.08	3.98	4.7 ± 0.09	69 ± 0.54	53 ± 0.81	18 ± 0.59	26 ± 0.83	56 ± 0.26
12	160	10	0.08	2.77	5.4 ± 0.09	78 ± 0.90	49 ± 0.62	25 ± 0.70	24 ± 0.67	37 ± 0.48
13	160	30	0.12	3.24	5.1 ± 0.11	75 ± 0.65	52 ± 0.63	22 ± 0.85	24 ± 0.35	47 ± 0.62
14	185	50	0.04	4.20	4.2 ± 0.05	57 ± 1.20	64 ± 0.99	1 ± 0.10	32 ± 0.84	51 ± 1.29
15	185	30	0.08	3.98	4.5 ± 0.08	69 ± 1.10	53 ± 0.63	17 ± 0.56	26 ± 0.96	58 ± 1.68
16	210	30	0.12	4.72	3.7 ± 0.11	54 ± 1.20	63 ± 0.88	0 ± 0.00	33 ± 0.68	48 ± 0.87
17	185	10	0.12	3.50	5.0 ± 0.14	72 ± 0.75	52 ± 0.74	20 ± 0.77	25 ± 0.49	51 ± 1.28

Footnotes: Tem, temperature; T, time; SF, severity factor (log R_0); SR, solid recovery rate; Glu, glucan; Xyl, xylan; Lin, lignin; SY, sugars yield;

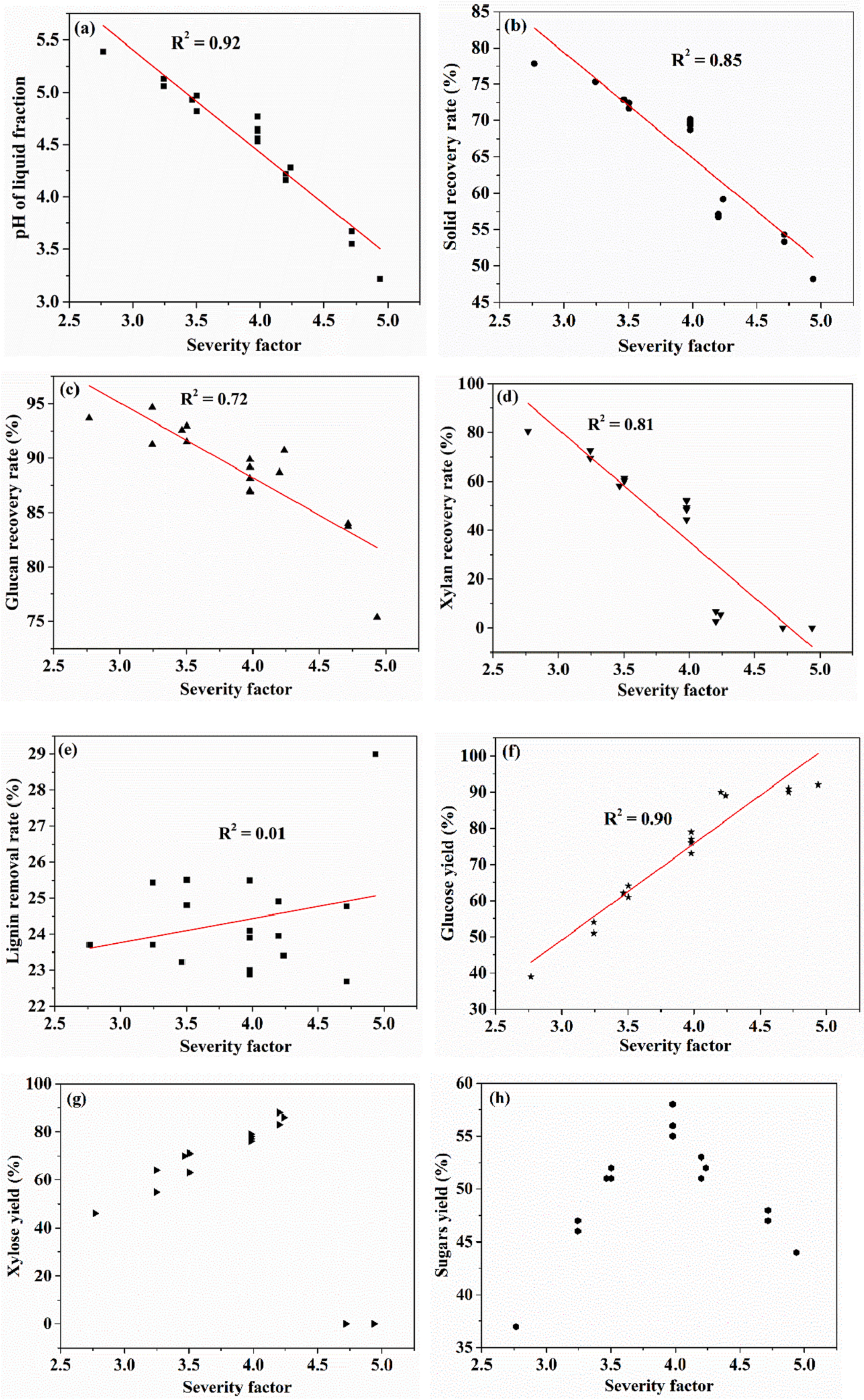

Fig. 1. The relationship between the effects of pretreatment/ enzymatic hydrolysis and severity factor.

Table 3
The variance for the regression quadratic model equation of central composite Design.

Source	Sum of squares	Degree of freedom	Mean square	F value	P-vale Prob > F	significant
Model	443.06	9	49.23	32.77	< 0.0001	significant
A-Tem	15.28	1	15.28	10.17	0.0153	
B-Time	5.75	1	5.75	3.83	0.0913	
C—S: L	0.47	1	0.47	0.31	0.5922	
AB	133.84	1	133.84	89.11	< 0.0001	
AC	0.11	1	0.11	0.073	0.7948	
BC	1.20	1	1.20	0.80	0.4015	
A^2	225.10	1	225.10	149.86	< 0.0001	
B^2	29.28	1	29.28	19.49	0.0031	
C^2	13.12	1	13.12	8.73	0.0212	
Residual	10.51	7	1.50			
Lack of Fit	6.78	3	2.26	2.42	0.2060	not significant
Pure Error	3.73	4	0.93			
Cor Total	453.57	16				

$R^2 = 0.9768$; Adjusted $R^2 = 0.9470$
C.V. = 2.43 %; Adequate precision = 19.110
Predicted residual sum of squares = 114.36

3.2. *Optimization of liquid hot water pretreatment approach via response surface methodology*

The results of optimization of liquid hot water pretreatment of SCB are presented in Table 3. "*P*-value prob > F" of A, AB, A^2, B^2 and C^2 below 0.05 reveled these factors were significant model terms; *P*-value < 0.01 and *F*-value = 32.77 implied the regression model was significant and hardly interfered by noise (the probability below 0.01 %); both the *F*-value and *P*-value of "lack of fit" much >0.05 suggested that the pure error of this model was not significant; furthermore, the chance of the accuracy of model interfered by noise was at very low level due to its signal-to-noise ratio > 4 (Kang et al., 2020); and the coefficient variation (CV) = 2.43 % reveled that this model can navigate the experimental design accurately (Zhang et al., 2022). Coefficient of determination (R^2) is generally applied to assess the accuracy of model fitting, the adjusted $R^2 = 0.9470$ indicates that the established model can interpret 94.7 % accurate in sugars yield response to pretreatment conditions changes (Zhang et al., 2022). The value of predicted residual sum of squares high to 114.36 implied that the established model could well fit the experimental data points. Multinomial regression analysis was performed according to the experimental results, after the elimination of insignificant variables ($p > 0.05$) that affecting the response sugars yield by successive iterations, the correlations between different pretreatment conditions and sugar yield is showed in Eq. (7). Among the different independent variables, the pretreatment temperature has the greatest influence on the sugar yield, followed by the pretreatment time, and the solid–liquid ratio has a very limited influence.

$$\begin{aligned} SY(\%) = & -442.81937 + 4.78269A + 3.42325B + 190.41735C \\ & -0.015426AB - 0.16559AC + 0.91208BC - 0.011699A^2 \\ & -0.01172B^2 - 1103.15949C^2 \end{aligned}$$

The 3D response surface and contour map of different independent factors affecting sugar yield are illustrated in Fig. 2. The combined effects of pretreatment temperature (160–210 °C) and time (10–40 min) on sugars yield are showed in Fig. 2(a) and (b). The similar 3D response surface shape and apex height between prolong low temperature pretreatment time and pretreatment at high temperature for short time. The obtained sugars yield was <50 % even extending the reaction time to 40 min at temperature below 180 °C; the sugars yield of over 55 % could be achieved when pretreatment was conducted at 190 °C for no <15 min; although continuing to increase the pretreatment temperature to over 200 °C help to remove more barriers and boost enzymatic hydrolysis, it would lead to a large amount of carbohydrate loss, which is not conductive to obtain high sugars yield.

The interaction effects of solid-to-liquid ration on sugars yield are showed in Fig. 2(c) and (d). The arched three-dimensional response surface and oval contour (the sugars yield first increased and then decreased as the temperature increased from 160 to 210 °C) of the interaction of these factors were observed, with the rose of pretreatment temperature, the response surface exhibited a steep shape (rising or falling), indicating the sugars yield was greatly affected by temperature, and the vertex appeared when the pretreatment temperature was between 180 and 190 °C, while the change in solid-to-liquid ratio showed limited effect on sugars yield. On the one hand, increasing the pretreatment temperature with a certain range favor to remove more hemicellulose, facilitating the enzymatic hydrolysis of pretreated solid residual and enhance sugars yield; on the other hand, excessive temperature can lead to extensive hydrolysis and conversion of carbohydrates, thereby reducing sugars yield.

The combined effects of S: L and reaction time on sugars yield are illustrated in Fig. 2(e) and (f). The flat 3D response surface and small fall between the contour lines indicates that the changes in solid–liquid ratio and the reaction time exhibited little effect on the sugars yield. The reaction time exhibited greater impact on the sugars yield than S: L, moderately increasing the substrate content combined with prolonging reaction time would not affect sugars yield, in other words, the efficiency of reaction equipment has enhanced. Too high substrate content leads to high viscosity, while too long reaction time leads to carbohydrates loss, pretreatment of SCB with substrate loading of 7–9 % (w/v) for 25–30 min favor to achieve high sugars yield.

The above results show that different independent variables affect sugars yield interactively within a certain range, and the predicted highest sugars yield high to 56.1 % could be achieved at the conditions of temperature 186.14 °C, time 26.77 min and S: L 0.08. Based on the optimized results, pretreatment of SCB were performed following different procedures to validate the established model, the chemicals components and enzymatic hydrolysis efficiency of pretreated solid residuals, the pH value and the concentrations of furan derivatives, acid, monosaccharide in pretreatment solution were investigated. After pretreatment of SCB raw materials via the processes 1–5 and followed by enzymatic hydrolysis for 72 h, the sugars yield of 51 %, 57 %, 54 %, 50 %, and 51 % were achieved, which were consistent with the predictions of established 3D response surface regression models. Pretreatment of sugarcane bagasse at 175–200 °C for 20–35 min mainly remove hemicellulose, but beyond the thresholds will lead to the remove of cellulose and generate a large amount of potential inhibitory derivatives (such as furfural, hydroxymethyl furfural, AA, FA, and levulinic acid, etc.). Making fully utilization of the fermentable sugars in the pretreatment solution conducive to reducing the nutrients and reduce the risk of eutrophication caused by the discharge of the waste liquid, however, the furan derivatives generated from lignocellulose during pretreatment procedure possibly cause inhibitory effects on the growth and metabolism of many microorganisms (Rahmani et al., 2022). Therefore, optimization of the temperature and time for LHW pretreatment is crucial to maximum the utilization of carbohydrates in lignocellulosic biomass and construct clean bio-refinery process, and the pretreatment condition selected in this study was temperature 186 °C, time 27 min and S:L 0.08.

3.3. *Structural characterization*

SEM analysis found that the raw material was in the form of flakes, with a smooth surface and a dense structure. After pretreatment, the smooth surface morphology of raw material was destroyed due to the

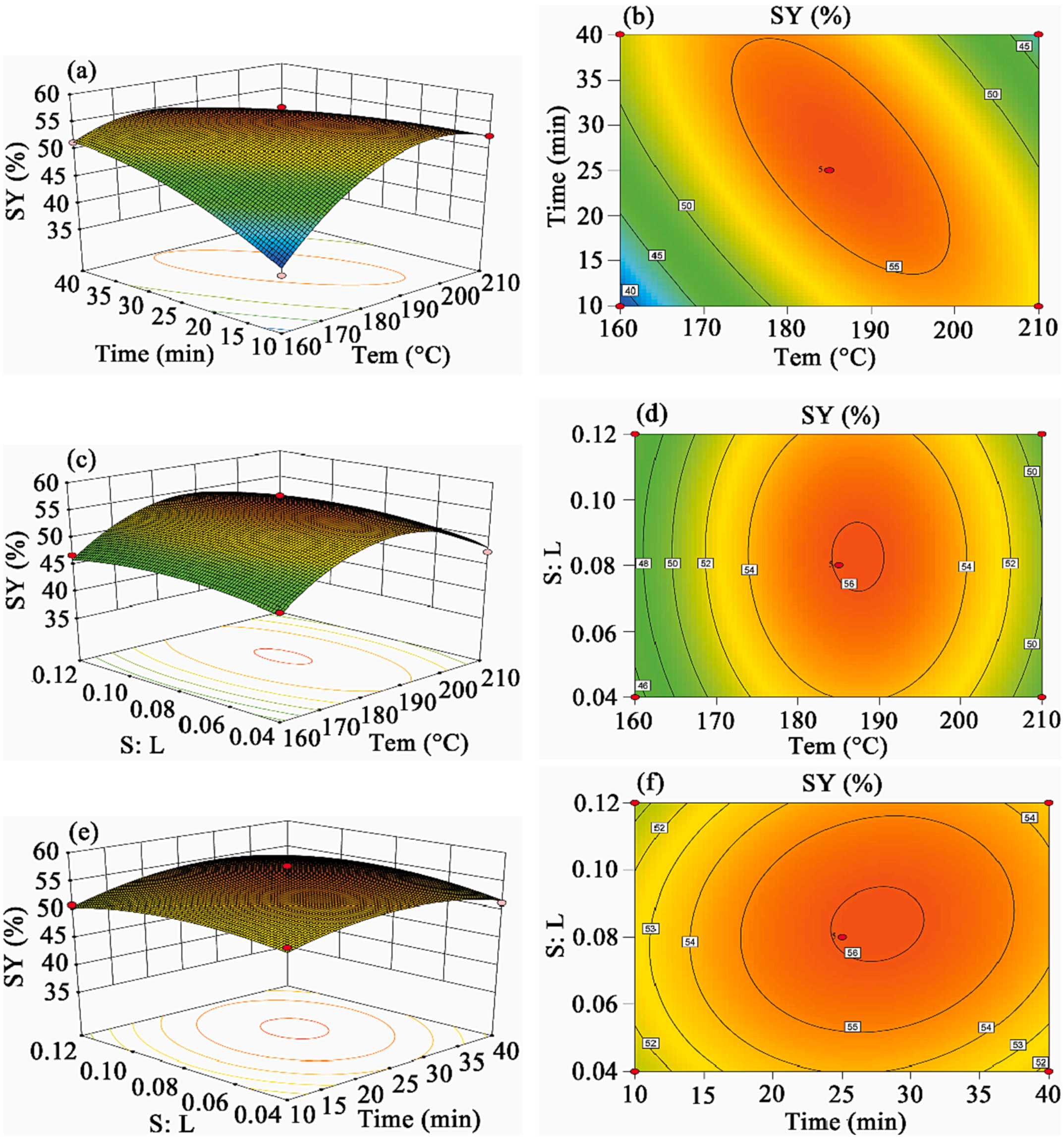

Fig. 2. Response surface analysis of sugars yield obtained from the liquid hot water pretreated sugarcane bagasse.

removal of some chemical components (especially hemicellulose), the exposure of fibers results in a large number of groove-like structures presented on the surface. The destruction of the original structure of raw material favors to increase its surface area and improve the accessibility of the cellulose to cellulase, thus boosting its enzymatic saccharification.

The effect of liquid hot water pretreatment on the structure and chemical bonds of different feedstocks were analyzed by comparing their infrared spectrogram. The main elements in lignocellulose are C, H, and O, and the infrared spectra representing the chemical bonds between them were analyzed by referring to the corresponding publications (Yuan et al., 2019). The stretching and deformation vibration of C—H bonds in the methyl, methylene and syringl are appeared at 1462 cm^{-1}, 2943 cm^{-1} and 825 cm^{-1}. The peak of the C═O rocking vibration and/ or C—O stretching vibration occurs at 1257 cm^{-1}, the C—O deformation vibration in secondary alcohols or aliphatic ethers appears at 1060 cm^{-1}, the peak located at 1159 cm^{-1} is attributed to the aliphatic C═O stretching vibration and C—O—C stretching vibration in pyran ring. The O—H bond in cellulose can be characterized by the peak at 3423 cm^{-1}. The vibration of aromatic rings in lignin causes infrared absorption peaks at 1515 cm^{-1} and 1600 cm^{-1}. Compared with the raw material, the change of the characteristic peaks of lignin in the pretreated residue indicates that the subunit structure of lignin was changed during the pretreatment process, while the increase in the intensity of the peaks representing cellulose indicates that the cellulose content in the feedstock was increased.

XRD analysis was performed to further explore the mechanism of enhancing enzymatic hydrolysis efficiency of SCB by liquid hot water pretreatment. The main peaks of different samples were located at 22°, exhibiting typical characteristics of cellulose I, revealing that the crystalline form of cellulose did not change during pretreatment process (Jin et al., 2016). After liquid hot water pretreatment, the crystallization index of SCB was increased from 48.1 % to 61.8 %, indicating the pretreatment procedure has increased the cellulose content, which was consistent with the results of chemical components analysis.

3.4. *Effects of solid content and cellulase addition amount on enzymatic hydrolysis*

It is well known that expensive cellulase is not conducive to large-scale biorefining of lignocellulose, therefore, it is critical to give full play to the catalytic effect of cellulase and reduce its dosage (Baral et al., 2022). The effects of different cellulase dosage (10, 20, 30, 40 FPU/g) and substrate loading (5 %, 10 %, 15 %, 20 % (w/v)) on sugars yield of LHW pretreated solid were evaluated, and the results are showed in

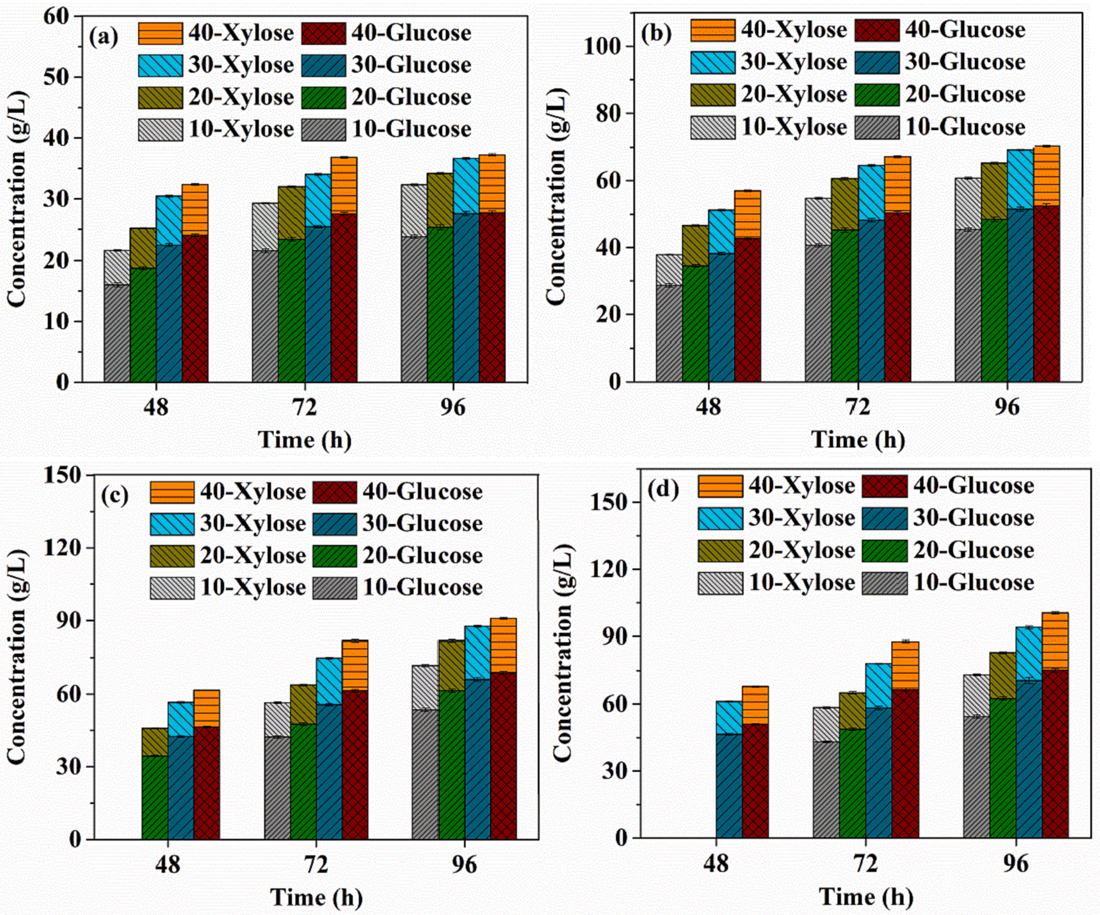

Fig. 3. Effects of different cellulase loading (10, 20, 30, 40 FPU/g) on enzymatic hydrolysis with various substrate contents (5 %, 10 %, 15 %, 20 %).

Fig. 3. The sugars yield rapidly increased as the cellulase dosage increased, especially in the process of high-solids enzymatic hydrolysis, which could be attributed to increase the amount of cellulase helps to accelerate the hydrolysis of cellulose and the dissolution of solid substrates, thus improving the flow performance of substrate and boosting the enzymatic digestion. When the substrate content was below 10 % (w/v), performing enzymatic digestion with cellulase loading of >30 FPU/g DM for 72 h, the achieved sugars yield over 80 %. For conducting enzymatic digestion at 20 % (w/v) substrate content, the obtained yield and concentration of sugars only approximately 60 % and 123 g/L even the cellulase dosage increased to 40 FPU/g DM and time prolonged to 96 h. It was revealed that performing high-solids enzymatic hydrolysis using batch strategy incapable to achieve high efficiency even at high cellulase loading. When implementing enzymatic hydrolysis at high-solids content, adding substrates in batches favor to overcome the drawbacks such as lack of free water, high viscosity, low mass and heat transfer efficiency, thus enhancing saccharification efficiency (Xu et al., 2019a). Comprehensive consideration of enzymatic hydrolysis efficiency and cost of cellulase, the cellulase loading of 20 FPU/g substrate was selected for further research.

3.5. *Effect of washing step on enzymatic hydrolysis*

In general, to remove the potential inhibitory derivatives produced during pretreatment process, the step of washing pretreated residue was introduced before enzymatic hydrolysis (Chen et al., 2021). However, one the one hand, the washing step would consume large amount of clean water and the discharge of waste water will cause environmental pollution; on the other hand, during the pretreatment process, many hemicellulose and little cellulose would dissolve in pretreatment liquid, thus the discharging leads to waste of carbohydrates and decrease in the efficiency of biorefinery. The effect of washing step on the enzymatic digestion of LHW pretreated residue was evaluated, the results showed that the sample underwent washing step help to accelerate the initial enzymatic hydrolysis (<24 h), however, with the extension of digestion time, there was no significant difference in the sugars yield between washed and unwashed substrates. This may be attributed to the lignocellulosic derivatives in the pretreatment liquid may have toxic effects on the activity of the cellulose. In addition, the solids in the pretreatment liquid may cause non-productive adsorption to cellulase, which have greater influences on the free movement of cellulase than the inhibition of its activity.

3.6. *Effects of hemicellulase and sophorolipids on enzymatic hydrolysis*

The previous study has proved that both addition of hemicellulase and sophorolipids are favor to enhance the saccharification efficiency of lignocellulosic feedstock (Xu et al., 2019a), and the effects of them on enzymatic digestion of LHW pretreated SCB are presented in Fig. 4. Cellulase and hemicellulase exhibited synergistic effect on enzymatic hydrolysis of feedstock, as compared to control (without addition of hemicellulase), the concentrations of glucose and xylose were increased by 4.35 g/L and 7.6 g/L with hemicellulase addition amount of 600 U/g DM, while the addition of more hemicellulase will not further enhance the enzymatic hydrolysis efficiency. Increasing the hemicellulase dosage mainly enhances sugars yield from the following aspects (Batista et al., 2019): (1) it helps to accelerate the hydrolysis of hemicellulose in the solid fraction to release sugars, while eliminating the barrier of hemicellulose on cellulase hydrolysis; (2) it favors to digest xylan oligosaccharide in the pretreatment liquid and reduce its inhibition on the

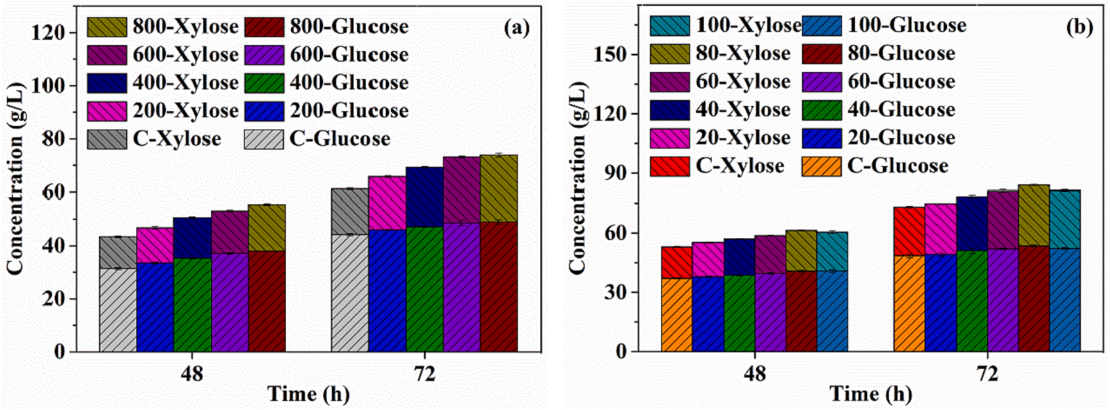

Fig. 4. The effect of hemicellulase dosage (a) (0, 200, 400, 600, 800 U/g DM) and sophorolipids addition amount (b) (0, 20, 40, 60, 80, 100 mg/g DM) on the enzymatic hydrolysis of without washed substrates (10 % solid content).

enzymatic hydrolysis. The presence of sophorolipids also favor to boost enzymatic hydrolysis, as the addition amount increased from 20 to 100 mg/g DM, the total sugar concentration exhibited an overall upward trend, however, with the increase of the added amount, the promotion effect gradually decreases. The highest total sugars concentration was achieved with sophorolipid addition amount of 80 mg/g DM, which was 13.07 % higher than the control (without addition of hemicellulase). Sophorolipid, as a biosurfactant, has been proved to interact with lignin to change its surface properties, such as reducing hydrophobicity and surface potential, thereby reducing the non-productive adsorption of cellulase by lignin; moreover, the addition of sophorolipid into enzymatic hydrolysis system favor to improve the stability of cellulase under harsh conditions such as high temperature and mechanical shearing, therefore boosting enzymatic hydrolysis (Xu et al., 2021b).

3.7. *High-solids enzymatic hydrolysis via different feeding strategies*

The effects of different feeding strategies on enzymatic digestion of LHW pretreated SCB are presented in Table 4. As compared with batch mode, the concentration glucose and xylose were obviously increased with the help of fed-batch strategies, this could be ascribed to the fed-batch modes can maintain the actual solids content at low level by adding the substrate in batches, thereby alleviating the adverse effects of high viscosity, water shortage, etc. When preforming high-solids content enzymatic hydrolysis with fed-batch strategy, addition of solid substrate from high to low helps to increase the efficiency of enzymatic hydrolysis. This is due to the cellulase activity at high level in the initial stage, which would favor to rapidly liquefy feedstocks and thereby alleviating the adverse effects of low heat and mass transfer efficiency caused by high solid content (Xu et al., 2019a). When the LHW pretreated residual was enzymatic digested at 20 % (w/v) solid content following the feeding strategy of adding 6 %, 5 %, 4 %, 3 % and 2 % (w/v) solid at 0 h, 6 h, 12 h, 18 h and 24 h, the highest concentrations of 90 g/L glucose and 31 g/L xylose could be achieved, which was 45.78 % higher than batch mode. Enzymatic digestion of lignocellulosic feedstocks at >15 % (w/v) solid content favor to achieved high fermentable sugars concentration, therefore saving energy, water and downstream processing cost, which exhibited high application values in improving the economic and environmental benefits of biorefinery (Baral et al., 2022; Xu et al, 2019a). However, this approach faces the challenge of low saccharification efficiency caused by the negative effects of high solid loading, the fed-batch strategy can help to overcome these difficulties by maintaining low solids content in the enzymatic hydrolysis system, furthermore, this method is easy to operate and no need to improve the equipment (Shiva et al., 2022). He et al. (2018) performed enzymatic hydrolysis of pretreated lignocellulosic feedstock via fed-batch and batch strategies, as the substrate dosage increased from 5 % to 15 % (w/v), the obtained sugars yield via former was superior to later. Previous study using alkali to pretreatment SCB, followed by enzymatic digestion the residual at 22 % (w/v) substrate content via fed-bath and batch modes, the achieved sugars yield via the former strategy was 13 % higher than that of the latter (Xu et al., 2019a).

3.8. *Clean procedure for SA fermentation*

Only very limited derivatives (such as furfural, hydroxymethyl furfural, etc.) with potential inhibitory effect on microbial fermentation were formed during the pretreatment process of approach 2, and some fermentable sugars were detected in the pretreatment liquid. Therefore, adding the pretreatment solution into the fermentation broth for SA fermentation would favor to both reduce the discharge of waste liquid and make full utilization of fermentable sugars, the effects of different addition amount of pretreatment liquid on SA fermentation were evaluated. The results showed that although SA conversion rate slightly decreased as the addition amount of pretreatment liquid increased from 0 to 60 % total volume of the fermentation broth, the obtained total SA

Table 4
Enzymatic hydrolysis of without washed pretreated solid at high-solids content.

	Enzymatic hydrolysis												
	Feeding strategy (h, %)					72 h				96 h			
Code	0	6	12	18	24	Glucose C (g/L)	Y (%)	Xylose C (g/L)	Y (%)	Glucose C (g/L)	Y (%)	Xylose C (g/L)	Y (%)
1	20					48 ± 0.99	40 ± 0.83	17 ± 0.44	41 ± 1.09	62 ± 0.87	52 ± 0.73	21 ± 0.66	53 ± 1.63
2	8	6	4	2		69 ± 0.30	58 ± 0.25	23 ± 0.36	58 ± 0.88	76 ± 0.89	64 ± 0.74	26 ± 0.74	64 ± 1.83
3	7	5	4	2	2	78 ± 0.46	65 ± 0.39	27 ± 0.60	65 ± 1.45	84 ± 0.51	70 ± 0.43	29 ± 0.48	71 ± 1.19
4	6	5	4	3	2	85 ± 0.91	71 ± 0.76	28 ± 0.36	69 ± 0.88	90 ± 0.94	76 ± 0.79	31 ± 0.43	76 ± 1.07
5	5	5	5	5		72 ± 1.34	61 ± 1.12	25 ± 0.49	61 ± 1.22	81 ± 1.37	68 ± 1.15	27 ± 0.36	66 ± 0.89
6	5	5	4	3	3	83 ± 0.75	69 ± 0.63	26 ± 0.73	63 ± 1.79	87 ± 0.89	73 ± 0.74	29 ± 0.85	71 ± 2.10
7	4	4	4	4	4	84 ± 0.71	70 ± 0.60	28 ± 0.68	70 ± 1.66	88 ± 0.60	73 ± 0.51	30 ± 0.81	74 ± 2.00

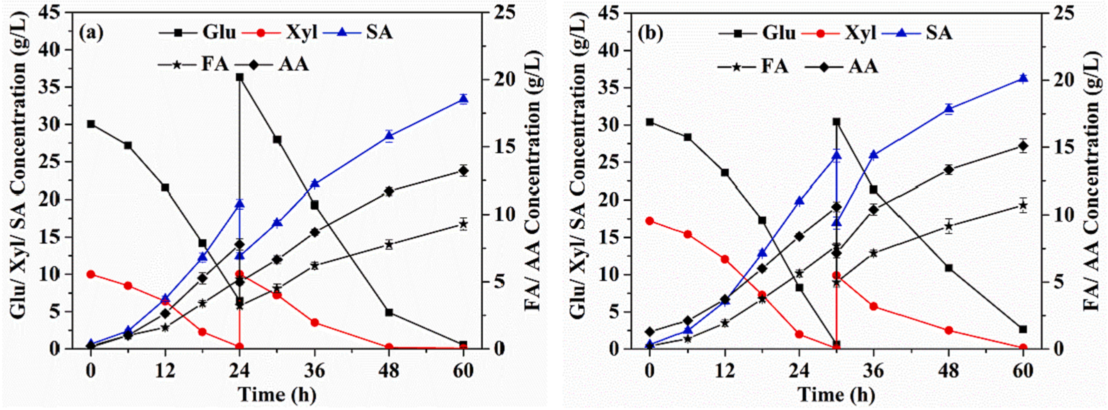

Fig. 5. The effect of pretreatment solution on fed-batch fermentation of succinic acid. (a) the enzymatic hydrolysate of pretreated SCB (washed) was used as carbon source, no pretreatment liquid was reused during fermentation process; (b) the enzymatic hydrolysate of pretreated SCB (unwashed) was used as carbon sources, the pretreatment liquid was partial replenished into broth during fermentation process.

concentration did not obviously decrease ascribed to the fermentable sugars in the pretreatment liquid were utilized for SA production.

In order to overcome the disadvantages of high glucose concentration inhibiting the metabolic capacity of *A. succinogenes* ATCC 55618, the fed-batch fermentation strategy was applied for SA fermentation, to reduce water consumption and waste liquid discharge, the effects of washing pretreated residue and without adding pretreatment liquid into fermentation broth (a) or not (b) on SA yield were evaluated, and the results are illustrated in Fig. 5. For approach (a), 50 mL hydrolysate was used as carbon source at initial fermentation stage (the concentrations of glucose and xylose were approximately 30 g/L and 10 g/L), then 90 mL hydrolysate was fed at 24 h, after fermentation for 60 h, almost all of glucose and xylose were consumed, and the SA concentration reached to 33.36 g/L. For procedure (b), in specific, the fermentation medium was prepared with pretreatment liquid instead of water, 50 mL and 90 mL of pretreatment solution were added at the initial stage and fermentation for 30 h, as compared to approach (a), the SA productivity was much lower in (b) at initial stage, and this difference decreased as fermentation time prolonged, after fermentation for 60 h, the glucose and xylose were almost completely converted to SA, FA, AA and other products, and the achieved SA high to 36.24 g/L, which was 8.6 % higher than that of approach (a). The FA and AA in fermentation broth mainly formed from the processes of microbial fermentation and liquid hot pretreatment, and their contents are positively correlated with succinic acid content to a certain extent. In the process of microbial fermentation, although the synthesis pathways of SA, FA and AA will compete carbon source, the reduction forces generated during the synthesis of FA and AA are necessary for synthesis of SA (Xu et al., 2022). Therefore, in the follow-up study, it can be considered to appropriately regulate the synthesis flux of FA and AA through genetic modification and fermentation condition control to enhance succinic acid yield. The above results indicated that adding pretreatment liquid into fermentation broth can not only reduce washing water consumption and waste liquid emission, but also help to enhance SA conversion rate.

3.9. Mass balance

Mass balance of the approach of SA processed from SCB was conducted to access the efficiency, environmental and economic benefits of developed technology (presented in Fig. 6). Compared with the conventional approach (washing pretreated residue before enzymatic hydrolysis and without addition of pretreatment liquid into SA fermentation broth), the established process exhibited obvious advantages in obtaining SCB raw material – to – SA conversion rate, reducing water consumption and waste liquid emission, etc. The optimized method was applied to pretreat 100 g SCB raw material, after

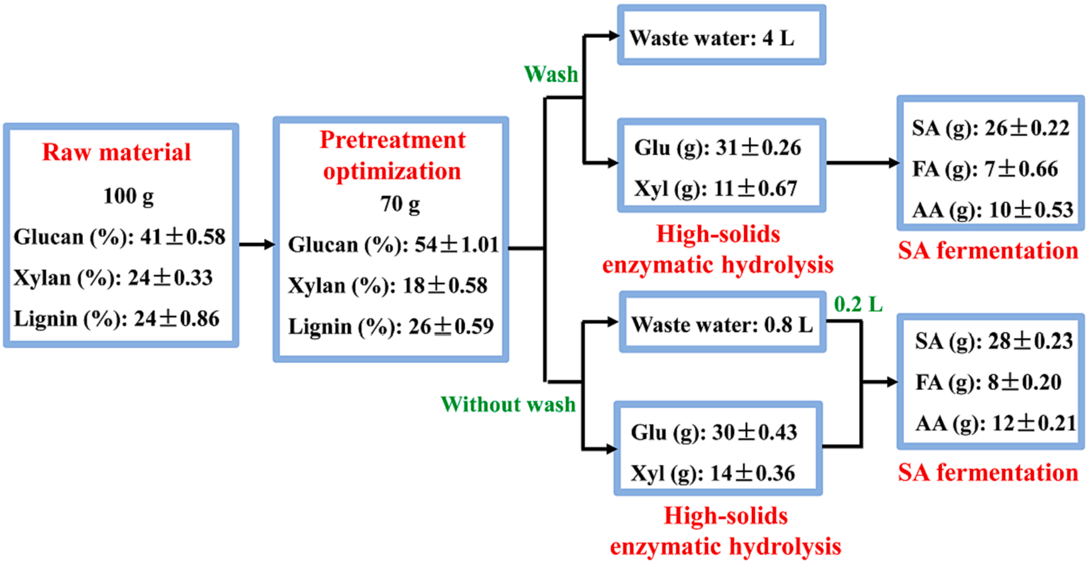

Fig. 6. Mass balance. Footnotes: FA, formic acid; AA, acetic acid.

solid–liquid separation, approximately 70 g solid residue and 0.8 L pretreatment liquid were collected. When processing SA via common process, that is, washing the pretreated residue with tap water, followed by enzymatic digestion and SA fermentation, the discharged wastewater would over 4 L and the achieved SA, AA and FA reach to 26, 10 and 7 g, respectively. As the SA was processed following the established technology of present study, the without washed pretreated residue was enzymatic digested at 20 % (w/v) solid content, and pretreatment liquid was added in the SA fermentation broth to make full utilization of fermentable sugars and reduce waste liquid emission, the discharged pretreatment liquid was reduced to 0.6 L, the achieved mass of SA, AA and FA were increase to 28, 12 and 8 g, respectively.

In recent years, the use of lignocellulose as raw material for the production of bio-based chemicals has attracted extensive attention (Lu et al., 2021). Many researchers have tried to process bio-SA from lignocellulosic biomass, Lo et al. (2020) applied the phosphoric acid to pretreat sweet sorghum bagasse, followed by enzymatic hydrolysis and SA fermentation, the achieved concentration and conversion rate of SA were 17.8 g/L and 0.24 g/g DM, respectively. Olive pits and SCB were proved as excellent substrates for SA processing, 28–34 g/L SA with a conversion rate of 0.27 g/g DM could be obtained (Jokodola et al., 2022). The addition of glycerol into the hydrolysate of Napier grass helps to enhance the SA conversion rate, the yields of SA and SA/AA ratio were increased by 52 % and 41 % by compounding substrates and optimizing fermentation conditions (Lee et al., 2022). In this study, the SCB was pretreated by liquid hot water, followed by enzymatic hydrolysis and SA fermentation via fed-batch strategies, the SA concentration of 36.24 g/L with a conversion rate of 280 mg/g SCB raw material was achieved. It is worth mentioning that this procedure can not only fully utilize the carbohydrates in lignocellulosic feedstocks, but also chemicals free and no waste liquid emission during the pretreatment process, which can provide novel clue and technology references for clean and efficient processing of SA from lignocellulosic biomass.

4. Conclusion

Optimized LHW pretreatment conditions with RSM to avoid carbohydrate loss while improving the degradability of SCB, the sugars yield exhibited obvious positive correlation with SF and xylose removal rate within a certain range. Adding accessory enzymes, additives and applying fed-batch strategy favor to boost high-solids enzymatic hydrolysis. The PL showed limited inhibitory effect on SA fermentation, the reuse of PL favor to reduce clean water consumption, waste water emission and fully convert the fermentable sugars released from feedstock into SA, a SA yield high to 280 mg/g SCB raw material could be achieved via the established approach.

CRediT authorship contribution statement

Chao Xu: Project administration, Data curation, Writing – original draft, Writing – review & editing. **Yaru Xiong:** Conceptualization, Methodology, Visualization, Supervision. **Jun Zhang:** Resources, Funding acquisition, Project administration. **Kuntai Li:** Data curation, Writing – review & editing. **Saiyi Zhong:** Data curation, Writing – review & editing. **Shushi Huang:** Supervision, Validation. **Chunliang Xie:** Data curation. **Wenbing Gong:** Supervision, Validation. **Zuohua Zhu:** Supervision, Validation. **Yingjun Zhou:** Supervision, Validation. **Yuande Peng:** Project administration, Data curation, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2022.128389.

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