Biomass and Bioenergy 205 (2026) 108514

Contents lists available at ScienceDirect

Biomass and Bioenergy

journal homepage: www.elsevier.com/locate/biombioe

Enzyme-free re-hydrolysis of wheat straw residues upgrades the overall cellulose hydrolysis yield and intracellular products fermentations

Shinan Wu, Zhibin Li, Bin Zhang *, Jie Bao **

State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, 130 Meilong Road, Shanghai, 200237, China

ARTICLE INFO

Keywords:
Lignocellulose
Enzymatic hydrolysis
Enzyme-free re-hydrolysis
Lipid
Single-cell proteins

ABSTRACT

Separated saccharification and fermentation (SHF) of lignocellulose suffers from inhibitions of high-titer monosaccharides on cellulase activity during hydrolysis step. However, SHF operations are required for the production of intracellular products to avoid the challenges associated with separating cells from lignin residues. This study developed an enzyme-free re-hydrolysis strategy to enhance sugar recovery from wheat straw solid residues after conventional enzymatic hydrolysis. By leveraging the cellulase already adsorbed on the solid residues, approximately 50 % of the remaining cellulose was hydrolyzed without additional enzyme input. The liquid fraction from re-hydrolysis was separated and recycled as process water for next round of hydrolysis of fresh wheat straw, increasing the overall glucose yield to 85.3 ± 1.4 % - an improvement of approximately 8.0 %. After the biodetoxification of hydrolysate, the fermentation efficiencies of intracellular microbial lipid and single-cell proteins were increased by 17.6 % and 21.0 %, respectively. This closed-loop approach not only improves hydrolysis efficiency and product synthesis, but also minimized enzyme and water consumption, offering a cost-effective and scalable strategy for lignocellulose-based biorefining.

1. Introduction

Lignocellulosic biomass, the most abundant raw feedstock in nature, principally comprises cellulose, hemicellulose, lignin and ash [1]. Hydrolyzing the lignocellulosic biomass into monosaccharides by cellulase cocktail and then bio-transforming the sugars into biochemicals or biofuels are important parts of biorefining [2]. The enzymatic hydrolysis (saccharification) process is the most critical part of the lignocellulosic biorefinery process [3].

There are two main different configurations for lignocellulosic biorefinery, separated saccharification and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) processes [4]. An obvious advantage of SHF is the possibility to choose the optimal conditions for both enzymatic hydrolysis and fermentation steps as they are temporally and spatially separated [5]. Nevertheless, the major drawbacks of SHF are the feedback inhibition on cellulase activity by the accumulated mono- and di-saccharides and the chances of contamination, which can reduce the production yield [6]. To overcome these issues, SSF has been proposed, where the hydrolysis and fermentation occur in a single vessel, and the sugars are fermented immediately so that it alleviates the end-product inhibition and improves the hydrolysis efficiency [3,7]. The major drawback of SSF is finding the compromised conditions for both hydrolysis and fermentation. Moreover, the lignin cannot be separated before fermentation, so extra mixing is needed [8]. Both the biocatalyst and fermenting microorganism cannot be recycled during the SSF process [6]. Therefore, SSF does not apply to the fermentations of intracellular products such as microbial lipid, single-cell proteins (SCP), or polyhydroxyalkanoates because of the difficulties associated with lignin residue separation from the cell and intracellular products. In such cases, SHF is the only reasonable option for biorefinery fermentations.

Much effort is still needed to make the enzymatic hydrolysis involved in the SHF process more efficiency [2]. Various strategies have been proposed to enhance the enzymatic hydrolysis efficiency of lignocellulose in SHF process, such as new bioreactor or process designs [9–11], optimization of pretreatment process and cellulase mixture [12], addition of additives [13,14], etc. However, in many conventional hydrolysis processes, achieving a higher overall sugar yield often requires an increased cellulase dosage, which can further exacerbate the already significant cost contribution of enzymes. One method that can reduce

* Corresponding author.
** Corresponding author.
E-mail addresses: binzh@ecust.edu.cn (B. Zhang), jbao@ecust.edu.cn (J. Bao).

https://doi.org/10.1016/j.biombioe.2025.108514
Received 3 August 2025; Received in revised form 26 September 2025; Accepted 19 October 2025
Available online 22 October 2025

the cost of hydrolysis is to recycle the cellulase by either separating the enzymes from the solid or liquid phases, or recycling the solid and/or liquid phase directly [15]. Liquid recycling is generally combined with other process steps e.g., elution or concentration [16]. And the free enzyme recovered from the liquid showed low digestibility due to the loss of synergistic effect [17]. Direct recycling of enzyme-bound to the solid residues after enzymatic hydrolysis is an easier operation and more economical [18,19].

Qi et al. reported that dilute acid pretreated wheat straw showed lower hydrolysis yield, but adsorbed more cellulase on solid residues compared to dilute alkali treated sample [20]. Zheng et al. demonstrated the interaction force adsorption at molecular level and enzymatic activity between lignin and cellulase in acid pretreated sugarcane bagasse [21]. Considering the acid pretreated has been deemed economically feasible at a larger scale compared to other pretreatments [22], one possible way is to make use of the residual cellulase enzymes adsorbed on acid pretreated solid lignocellulose feedstock.

In this study, a cellulase-free re-hydrolysis strategy was proposed. This involves a second hydrolysis step of the solid residue obtained after the first enzymatic hydrolysis of acid pretreated wheat straw, without adding any new cellulase. The liquid fraction from re-hydrolysis was separated and recycled as process water for next round of hydrolysis of fresh wheat straw. Clarified hydrolysate containing mixed lignocellulose-derived inhibitors (furfural, 5-hydroxymethylfurfural, acetic acid, etc.) was biodetoxified by a unique fungi *Paecilomyces variotii* FN89. The biodetoxified hydrolysate was further used for microbial lipid and single-cell proteins by an engineered oleaginous yeast *Trichosporon cutaneum* MP11. The benefits of this method include (i) maximum hydrolysis of the unhydrolyzed cellulose component in solid residues without adding additional fresh cellulase; (ii) improvements in fermentable sugars yield and production efficiency; (iii) no significant fluctuation in hydrolysis solids loading, final sugars concentration and yield in repeated rounds of hydrolysis; (iv) no contamination during the hydrolysis owing to the presence of inhibitor cocktails which can be efficiently removed by the consequent submerged liquid biodetoxification; (iv) successful application for the production of intracellular microbial lipid and single-cell proteins. This method overcomes the limiting factors of low sugar yield and fermentation efficiency in SHF process, facilitating its industrial application.

2. Materials and methods

2.1. Feedstock

The raw wheat straw was collected and coarsely chopped on the farm (31°9′605″N, 118°12′709″E) in Nanyang city, Henan province, China, and then milled by a hammer crushing machine through a mesh of 10 mm in diameter. The raw wheat straw contained 34.55 % (w/w) of cellulose, 21.91 % (w/w) of xylan, 20.36 % (w/w) of lignin and 9.57 % (w/w) of ash based on dry weight according to the methods described by National Renewable Energy Laboratory (NREL) report [23].

The milled wheat straw was then pretreated on a pilot scale. The pretreatment was conducted at a high solid/liquid ratio of 2:1, 175 °C for 5 min. The acid dosage for pretreatment was determined by a base pH-approaching method to correct the fluctuating pretreatment efficiency by a simple titration procedure [24]. The dosage of sulfuric acid catalyst was 4.0–5.0 % (w/w) based on the dry weight of wheat straw. After pretreatment, the pretreated wheat straw was discharged from the bottom outlet of the reactor. No aqueous water was released during the pretreatment, because the pretreated feedstocks absorbed all the free water (both the acid catalyst solution and the condensed water from vapor steam). The pretreated feedstocks were in solid particles form (moisture ~50 % w/w), instead of slurry form. The pretreated wheat straw contained 298.4 mg/g of cellulose, 56.6 mg/g of xylan, 37.8 mg/g of glucose, 106.7 mg/g of xylose, 27.2 mg/g of acetic acid, 3.7 mg/g of 5-HMF, 4.0 mg/g of furfural, 465.5 mg/g of lignin, ash and other components based on dry weight.

Commercial cellulase Cellic CTec 3HS was purchased from Novozymes (Beijing, China). The filter paper activity (FPA) and protein content of cellulase was 220.2 FPU/mL and 90.1 mg/mL, respectively, according to the instructions. The reagents were purchased from Sinopharm Chemical Reagent Co., Shanghai, China.

2.2. Strains and culture

Trichosporon cutaneum MP11 strain (CGMCC 20481) was used for single-cell proteins and lipid production [25]. *T. cutaneum* MP11 was obtained from the wild-type strain *T. cutaneum* ACCC 20271 by long-term evolution combined with centrifugal enrichment [26]. *T. cutaneum* MP11 was preserved on yeast extract peptone dextrose (YPD) plates. The nutrient for single-cell proteins contained 1 g/L of KH_2PO_4, 1 g/L of $MgSO_4{\cdot}7H_2O$, and 24 g/L of $(NH_4)_2SO_4$. The nutrients for lipid production contained 1.0 g/L of KH_2PO_4, 0.5 g/L of yeast extract, 0.5 g/L of $MgSO_4{\cdot}7H_2O$, and 0.22 g/L of $(NH_4)_2SO_4$.

Paecilomyces variotii FN89 (CGMCC 17665) was used for the biodetoxification of wheat straw hydrolysate [27]. *P. variotii* FN89 was preserved on potato dextrose agar (PDA) plates. The seed culture contained 2 g/L of KH_2PO_4, 1 g/L of $MgSO_4{\cdot}7H_2O$, 1 g/L of yeast extract, 1 g/L of $(NH_4)_2SO_4$, and 20 g/L of glucose. No nutrients were added during the biodetoxification.

2.3. Clarified wheat straw hydrolysate preparation

The pretreated wheat straw was neutralized by adding $CaCO_3$ powder. The saccharification was conducted in a 5-L bioreactor equipped with a helical impeller (BaoXing Bioengineering Equipment Co., Ltd., Shanghai, China). The saccharification lasted at 50 °C, 150 rpm for 24–72 h. The dosage of cellulase for saccharification was 2–4 mg protein/g substrate (dry weight). The solids loading was 30 % (w/w). The slurry was discharged from the bottom outlet of the bioreactor after saccharification, then centrifuged (Beckman J-26XP, Brea, CA) at 8000 rpm for 10 min to remove the solid residues. The supernatant of the slurry was collected and used for subsequent biodetoxification and fermentation.

2.4. Fresh cellulase-free re-hydrolysis of solid residues

The solid residues from wheat straw saccharification were collected by centrifugation, washed and used for re-hydrolysis. The re-hydrolysis of the solid residues was at 20 % (w/w) solids loading, 50 °C, 200 rpm for 12 h. No fresh cellulase was added because the solid residues adsorbed part of the cellulase during the saccharification. The hydrolysate of solid residues was centrifuged at 8000 rpm for 10 min, and then the supernatant was collected to replace part of the process water added in the next round of saccharification of wheat straw. The hydrolysate derived from the saccharification of pretreated wheat straw and the re-hydrolysis of solid residues was centrifuged to obtain the supernatant and solid residues. The supernatant was sent to biodetoxification and fermentation, and the solid residues were sent to the next round of re-hydrolysis, solid/liquid separation and saccharification.

2.5. Biodetoxification

The main inhibitors including acetic acid, 5-HMF and furfural in clarified wheat straw hydrolysate were removed by the unique biodetoxification strain *P. variotii* FN89. *P. variotii* FN89 was cultured in seed medium at 37 °C, 300 rpm for 24 h. The dry cell weight (DCW) of *P. variotii* FN89 in seed medium was approximately 0.5 g/L. The seed culture was inoculated to the hydrolysate at a ratio of 10 % (v/v). The biodetoxification was conducted at 37 °C, 750 rpm, 1.0 vvm until the pH of the hydrolysate started to decrease [27]. After the biodetoxification, the sodium hypochlorite (1.5 g/L) was added to the hydrolysate in order

to inactivate the biodetoxification strain.

2.6. Single-cell proteins and lipid fermentation

T. cutaneum MP11 was cultured on a YPD plate at 30 °C for 72 h. Then one colony was picked up, inoculated to 20 mL of fresh YPD liquid medium, and cultured at 30 °C, 200 rpm for 24 h. The culture broth was then inoculated into 100 mL of fresh YPD liquid medium at a ratio of 10 % (v/v), and cultured for 24 h as the seed. The seed was inoculated to the biodetoxified hydrolysate at the ratio of 10 % (v/w). Specific nutrient salt cocktails were added to regulate the strain's preference for the accumulation of single-cell proteins or lipid. The fermentation was conducted at 30 °C, 600 rpm, 1.0 vvm for 96 h. The fermentation pH was maintained at 5.0 by automatically adding 2 M H_2SO_4 and 5 M NaOH.

2.7. Yield and titer calculations

The yield of glucose was calculated according to Eq. (1) as follows:

$$\text{Glucose yield}\ (\%) = \frac{v \times ([glu]_1 - [glu]_2)}{s \times x_1 \times f_1} \quad (1)$$

where v (L) is the volume of the hydrolysate; $[glu]_1$ (g/L) is the concentration of glucose in the hydrolysate after saccharification; $[glu]_2$ (g/L) is the concentration of glucose in the slurry before saccharification; s (g) is the mass (dry weight) of the pretreated wheat straw used for saccharification; x_1 (%) is the content of cellulose in the pretreated wheat straw; f_1 is the coefficient for the conversion of cellulose to glucose, which is 1.11.

The titers of single-cell proteins and lipid were calculated according to Eqs. (2-3) as follows:

$$\text{Nitrogen content}\ (\%) : w = \frac{c \times (v_1 - v_2) \times 14 \times 6.25}{m} \quad (2)$$

where w (%) is the nitrogen content of the dry cell mass; c (mol/L) is the concentration of standard HCl solution for titration, which is 0.0993 mol/L; v_1 (mL) is the volume of standard HCl solution used for titration; v_2 (mL) is the volume of standard HCl solution used for titration of blank sample; 14 is the molar mass of nitrogen; 6.25 is the coefficient for the conversion of nitrogen to protein; m (g) is the dry cell weight.

$$\text{Single cell protein titer}\ (\text{g/L}) = \frac{w \times m}{v_3} \quad (3)$$

where w (%) is the nitrogen content of the dry cell mass; m (g) is the dry cell weight; v_3 (mL) is the volume of fermentation broth.

The yields of single-cell proteins and lipid were calculated according to Eq. (4) as follows:

$$\text{Product yield}\ (\text{g/g}) = \frac{v_4 \times c_1}{v_5 \times ([glu]_2 + [xyl]_2) - v_4 \times ([glu]_3 - [xyl]_3)} \quad (4)$$

where v_4 (mL) is the volume of final obtained fermentation broth; c (g/L) is the titer of single-cell proteins or lipid; v_5 (mL) is the volume of initial fermentation medium; $[glu]_2$ and $[xyl]_2$ are the concentrations of sugars in initial fermentation medium; $[glu]_3$ and $[xyl]_3$ are the concentrations of residual sugars in final obtained fermentation broth.

2.8. Analytical methods

The compositions of biomass and solid residues were determined according to the NREL report [23]. DCW was determined from the differences in weight of the dried centrifugation tube with and without cells. Lipid was extracted and quantified by chloroform-methanol method after the lysis of cells using a 4 M HCl solution. Glucose, xylose, acetic acid, 5-HMF, and furfural were determined by HPLC method [28].

3. Results and discussion

3.1. Saccharification of wheat straw at higher solids loading led to lower glucose yield

The saccharification of pretreated wheat straw was conducted at different solids loadings (15 %, 20 %, 25 %, 30 %, w/w) with the cellulase dosage of 4 mg protein/g substrate (equivalent to 10 FPU/g substrate) (Fig. 1). The glucose yield decreased significantly with higher solids content. When the saccharification was conducted at 15 % (w/w) and 20 % (w/w) solids loading, the glucose yield reached 96.2 ± 1.6 % and 86.3 ± 1.6 % at 48 h (Fig. 1c), while the total sugars (glucose and xylose) concentration was only 82.4 ± 6.2 g/L and 103.4 ± 1.0 g/L (Fig. 1a and b). In order to obtain higher concentration of fermentable sugars, the saccharification was conducted at 25 % (w/w) and 30 % (w/w) solids loading, while the glucose yield was only 79.0 ± 1.5 % and 79.6 ± 2.5 % (Fig. 1c). Over 95 % of free glucose was generated in the first 24 h of saccharification. Prolonging the saccharification period had limited effect on improving the glucose yield at high solids loadings.

The cellulase dosage for high-solids loading saccharification has been widely investigated. Ying et al. reported that the glucose yield was only about 50 % at 20 (w/w) solids loading with the cellulase dosage of 10 FPU/g substrate using acid-pretreated poplar, while the glucose yield was higher than 80 % with the cellulase dosage of 40 FPU/g substrate [29]. Unless the accessory enzymes and additives were added, the cellulase dosages in most studies were more than 10 FPU/g substrate [30]. Our previous study also indicated that 4 mg protein/g substrate (10 FPU/g substrate) is the lowest enzyme dosage for the saccharification of dry acid pretreated biomass [31,32]. The ineffective mass transfer has been considered as the major factor that leads to the decreased hydrolysis efficiency, particularly under high solids loadings [33]. While intensified mixing is commonly applied to mitigate mass transfer limitations, it may simultaneously alter other key factors influencing cellulose hydrolysis [34]. Vigorous mixing can accelerate the release of glucose, which at high concentrations severely inhibits cellulase activity. Operating batch hydrolysis under controlled mass-transfer-limited conditions can enhance sugar yields by reducing product inhibition [35].

3.2. Proof of the concept for re-hydrolysis of solid residues derived from saccharification

The reduction in glucose yield at higher solids loading indicated that there is a large amount of unhydrolyzed fractions in solid residues after saccharification. The contents of the residual cellulose and xylan components in solid residues were determined after 24 h's saccharification of pretreated wheat straw at solids loadings of 25 % (w/w) and 30 % (w/w) (Fig. S1a) (see supplementary materials). The xylose content in the solid residues is independent of the solids loading, which was less than 2 % (w/w) indicating almost all the xylan was hydrolyzed during the pretreatment and saccharification. But the contents of unhydrolyzed cellulose reached 7.6 ± 0.3 % and 8.0 ± 0.5 % in the solid residues after the saccharification at 25 % (w/w) and 30 % (w/w) solids loadings.

In addition to residual cellulose and xylan, the solid residues contained a large amount of lignin, insoluble ash and calcium sulfate [36,37]. The residual lignin can adsorb part or even most of the cellulase during the enzymatic hydrolysis through a synergistic effect of multiple factors, such as hydrophobic electrostatic, hydrogen-bonding interaction forces and enzyme surface polarity, etc. [21]. The solid residues were therefore further re-hydrolyzed at 20 % (w/w) solids loading for 12 h without adding fresh cellulase in order to improve the hydrolysis yield of cellulose. The results showed that about 50 % of the cellulose fraction in solid residues was further hydrolyzed during the re-hydrolysis (Fig. S1b) (see supplementary materials). Meanwhile, the hydrolysate derived from the re-hydrolysis of solid residues contained over 10 g/L of glucose, indicating that the adsorbed cellulase in solid

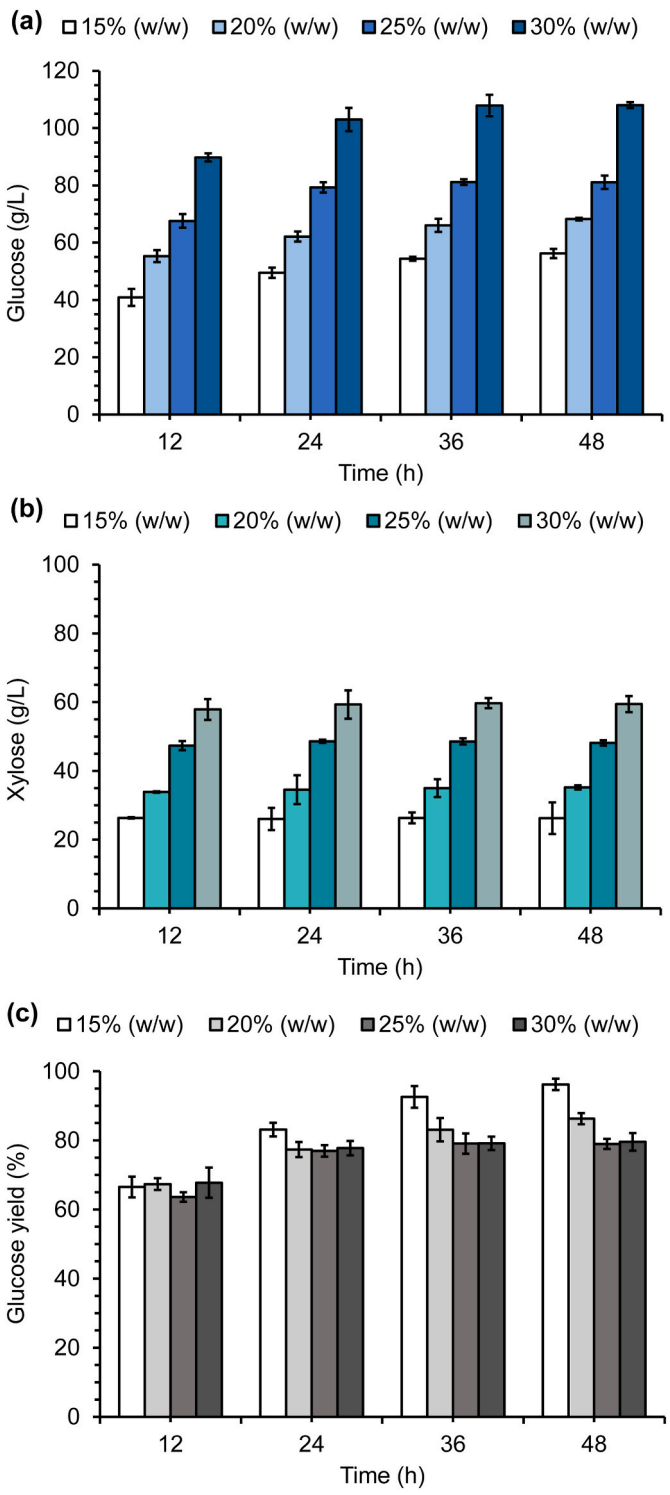

Fig. 1. Saccharification of dry acid pretreated wheat straw at different solids loadings (15–30 %, w/w) with the cellulase dosage of 4 mg protein/g substrate, equivalent to 10 FPU/g substrate. (a) Glucose concentration; (b) xylose concentration; (c) glucose yield. Saccharification conditions: 50 °C, 200 rpm, 48 h.

residues had the complete function to hydrolyze cellulose to glucose.

To utilize the sugars released from the re-hydrolysis of solid residues, the hydrolysate was collected and solid/liquid separated to obtain the supernatant. The supernatant was recycled as the process water for the next round of saccharification of pretreated wheat straw. This biorefinery process integrating the saccharification of pretreated wheat straw and the re-hydrolysis of solid residues is showed in Fig. S2 (see supplementary materials). The fermentable sugar concentrations and glucose yield were determined (Fig. 2). The sugar concentrations and glucose yield after the saccharification with the re-hydrolysis of solid residues were increased in all scenarios tested. The glucose concentration increased by 11.2 % and 9.7 % at the solids loading of 25 % (w/w) and 30 % (w/w) with the re-hydrolysis of solid residues, and the glucose yield increased from 78.4 ± 1.6 % and 77.7 ± 2.1 % to 87.5 ± 0.6 % and 85.3 ± 1.4 %, respectively. The total sugars (glucose and xylose) concentration reached 138.0 ± 1.8 g/L and 175.3 ± 4.2 g/L after the saccharification at the solids loading of 25 % (w/w) and 30 % (w/w), which were 7.8 % and 8.0 % higher than those without the re-hydrolysis of solid residues.

The recycling of the supernatant derived from the re-hydrolysis of solid residues would lead to an increase in the initial sugar concentration of the saccharification, which may have an impact on the yield of the next round of the saccharification. The stability of the saccharification of pretreated wheat straw with the re-hydrolysis of solid residues was further investigated. Two successive rounds of saccharification of pretreated wheat straw, solid/liquid separation, solid residues re-hydrolysis, and recycling of supernatant were carried out (Fig. 2). The results showed that after recycling of the supernatant derived from the re-hydrolysis of solid residues as saccharification process water, the total sugar concentrations were maintained at 137.5 ± 0.4 g/L and 175.5 ± 0.2 g/L at the solids loading of 25 % (w/w) and 30 % (w/w) in two

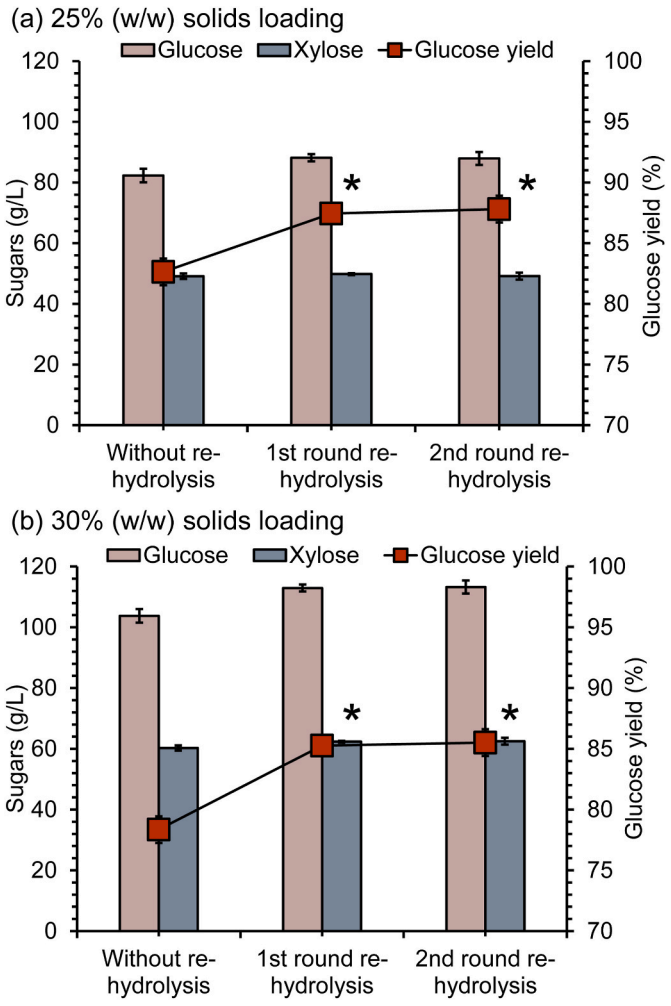

Fig. 2. Changes in sugar concentration and glucose yield after the saccharification with the re-hydrolysis of solid residues. (a) 25 % (w/w) solids loading; (b) 30 % (w/w) solids loading. Variations of glucose yield were considered statistically signifcant at *P < 0.05.

consecutive rounds of saccharification. The slight increase in the initial sugar concentration during the saccharification did not affect the glucose yield, which was maintained at 87.3 ± 0.1 % and 85.4 ± 0.1 % at the solids loading of 25 % (w/w) and 30 % (w/w) in two consecutive rounds of saccharification. These results not only demonstrated the good stability of this method, but also suggested that the saccharification yield at high solids loading was effectively improved by re-hydrolysis of the solid residues so that the glucose yield of saccharification at 30 % (w/w) solids loading was close to that at 25 % (w/w) solids loading.

3.3. Fermentability evaluation of the hydrolysate prepared by re-hydrolysis of solid residues

The hydrolysate contained various microbial growth inhibitors generated during the harsh pretreatment process, represented by acetic acid, 5-HMF, and furfural, which need to be removed prior to fermentation [38]. A submerged liquid biological detoxification (biodetoxification) method had been conducted in our previous study [27], which can efficiently remove these inhibitors while preserving the fermentable sugars. The hydrolysate prepared at 25 % (w/w) and 30 % (w/w) solids loading in this study contained 9.7 ± 0.2 g/L and 11.0 ± 0.2 g/L of acetic acid, 1.4 ± 0.1 g/L and 1.5 ± 0.1 g/L of 5-HMF, 1.3 ± 0.1 g/L and 1.6 ± 0.1 g/L of furfural, respectively (Fig. 3). The concentrations of 5-HMF and furfural in the hydrolysates with different solids loading (25 % and 30 %, w/w) were not significantly different, perhaps owing to their volatility and solubility. After the inoculation of the biodetoxification strain *P. variotii* FN89 to the hydrolysates, these three inhibitors were completely removed within 22 h with the loss of total fermentable sugars less than 1 %. Furfural and 5-HMF can severely inhibit the activity of glycolytic pathway [39]. *P. variotii* FN89 can convert these inhibitors to intermediates, and then entered the core carbon metabolism pathway for ultimate degradation and energy supply, which avoids the consumption of fermentable sugars [40]. *P. variotii* FN89 can efficiently remove furfural, HMF and acetate, but degrade phenolic aldehydes slowly [41]. Our previous study showed that after biodetoxification, the phenolic aldehydes were still in considerably high levels, with the representative phenolic compounds 4-hydroxybenzaldehyde (HBA), 4-hydroxy-3-methoxybenzaldehyde, syringaldehyde of 60.21 mg/L, 79.62 mg/L, 30.34 mg/L, respectively, and the total phenolics content of 690 mg/L [42]. *T. cutaneum* exhibited greater tolerance to these phenolic aldehydes than other oleaginous yeasts (e.g., *Rhodosporidium toruloides*, *Rhodotorula glutinis*, and *Yarrowia lipolytica*) [42], and was therefore selected for subsequent fermentation.

The biodetoxified hydrolysate was used for intracellular lipid and single-cell protein production by the engineered strain *T. cutaneum* MP11. Under the nitrogen-limited conditions, the oleaginous yeast tends to accumulate a large amount of lipid [43]. The dosage of ammonium sulfate was thus set at only 0.22 g/L for cellulosic lipid production (Fig. 4). All glucose and xylose were consumed by *T. cutaneum* MP11. The lipid titer reached 17.1 ± 3.1 g/L and 21.1 ± 1.1 g/L at 96 h using 25 % (w/w) and 30 % (w/w) solids loading hydrolysate by general separate hydrolysis and fermentation (SHF) process. The re-hydrolysis of solid residues improved the initial glucose concentration in hydrolysate, and the lipid titer reached 20.2 ± 0.9 g/L and 26.2 ± 2.0 g/L at 96 h using 25 % (w/w) and 30 % (w/w) solids loading hydrolysate, which were 16.6 % and 24.2 % higher than that in general SHF process.

Switching the nitrogen limitation condition to a nitrogen excess condition facilitated the biosynthesis of single-cell proteins by *T. cutaneum* MP11 [25]. The excessive 22 g/L of ammonium sulfate was added to redirect the biosynthesis from lipid to single-cell proteins (Fig. 5). The co-produced single-cell proteins and lipid in yeast can serve as a dual-nutrient components for poultry and aquaculture feeds [44–46]. The synthesis rate of single-cell proteins was higher than that of lipid during the early stages of fermentation. The highest single-cell protein

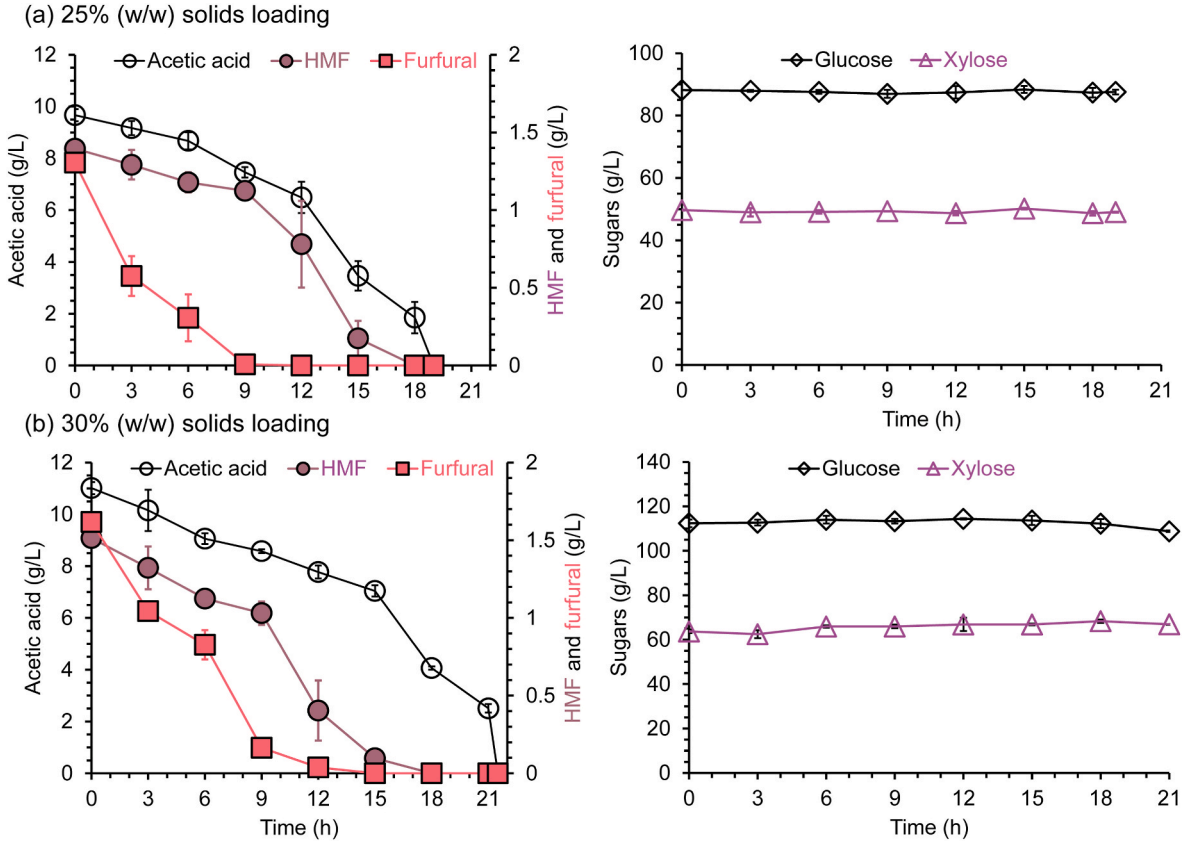

Fig. 3. Biodetoxification of clarified wheat straw hydrolysate. (a) The hydrolysate was prepared using 25 % (w/w) solids loading pretreated wheat straw; (b) the hydrolysate was prepared using 30 % (w/w) solids loading pretreated wheat straw. Conditions: 10 % (v/v) inoculation ratio, 37 °C, 750 rpm, 1.0 vvm.

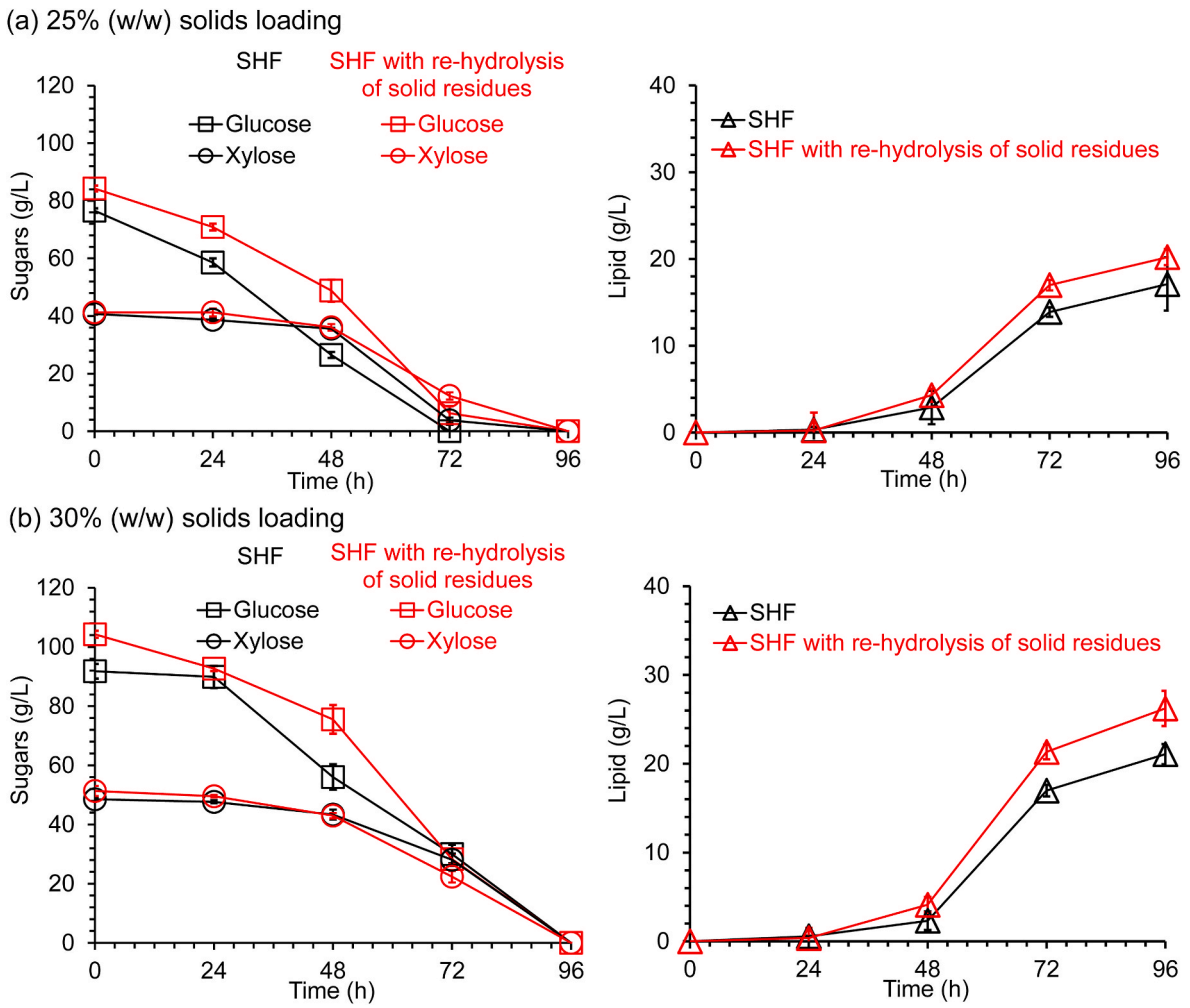

Fig. 4. Cellulosic lipid production using clarified wheat straw hydrolysate at a low concentration of nitrogen source. (a) 25 % (w/w) solids loading; (b) 30 % (w/w) solids loading. Conditions: 10 % (v/v) inoculation ratio, 30 °C, 600 rpm, 1.0 vvm, 0.44 g/L ammonium sulfate.

titer reached 13.1 ± 1.3 g/L and 16.7 ± 0.4 g/L at 72 h using 25 % (w/w) and 30 % (w/w) solids loading hydrolysate by the SHF process with the re-hydrolysis of solid residues, which were 25.2 % and 17.6 % higher than that in general SHF process. Then the single-cell protein titer declined after 72 h, and more lipid was accumulated. It was reported that disproportionately large levels of lipid accumulation in certain yeasts occurred at the expense of proteins and nucleic acids [47]. Furthermore, the culture aging and autolysis caused by nutrient depletion or higher initial inoculum also led to the decrease in single-cell protein production in the late stages of fermentation [48]. Finally, the lipid titer reached 20.9 ± 1.1 g/L at 96 h using 30 % (w/w) solids loading hydrolysate by the SHF process with the re-hydrolysis of solid residues, which was 31.8 % higher than that in general SHF process. The observed over 30 % increase in lipid and SCP production is disproportionate to the modest 9–12 % rise in glucose concentrations. The production increase is likely attributable to additional factors present in the re-hydrolysate, including (1) the presence of difficult-to-detect fermentable sugars in the hydrolysate (e.g., galactose, mannose, arabinose, xylo-oligosaccharides, or gluco-oligosaccharides), which may not be fully quantified by HPLC but could be utilized by *T. cutaneum* [26]; (2) the weak acids present in the hydrolysate (e.g., levulinic acid), which could serve as additional carbon sources or metabolic stimulants for fermentation; (3) variations in the concentration of soluble ions in the hydrolysate after re-hydrolysis, which might influence the fermentation performance and metabolic efficiency of the strain. Nevertheless, the exact mechanism remains unclear and warrants further systematic investigation.

3.4. Overall mass balance

To further characterize the effect of the re-hydrolysis of solid residues on fermentable sugars yield and production efficiency in biorefining process, the overall mass balances were calculated based on the aforementioned experimental results (Fig. 6). The saccharification and fermentation were conducted at 30 % (w/w) solids loading. The detailed material flows are shown in Fig. S3 (see supplementary materials).

The mass balance started from 100 kg (dry weight) of raw wheat straw, which contained 34.55 kg of cellulose and 21.91 kg of xylan. For the general SHF biorefining process (Fig. 6a), the raw wheat straw was pretreated, saccharified, and solid/liquid separated. The obtained clarified hydrolysate contained 21.21 kg of free glucose and 10.32 kg of free xylose. The solid residues contained 7.28 kg of unhydrolyzed cellulose, which accounted for 21.1 % of the cellulose mass in raw wheat straw. The solid/liquid separation was carried out in the laboratory by centrifugation, so that the moisture of solid residues was up to ~55 % (w/w), resulting in the loss of partial hydrolysate. The clarified hydrolysate was biodetoxified, and then used for lipid and single-cell protein production. 4.72 kg of lipid was produced under nitrogen-limited conditions, while 3.23 kg of single-cell proteins and 2.24 kg of lipid were coproduced under nitrogen-excessive conditions.

For the modified SHF biorefining process with the re-hydrolysis of solid residues (Fig. 6b), the supernatant derived from the re-hydrolysis

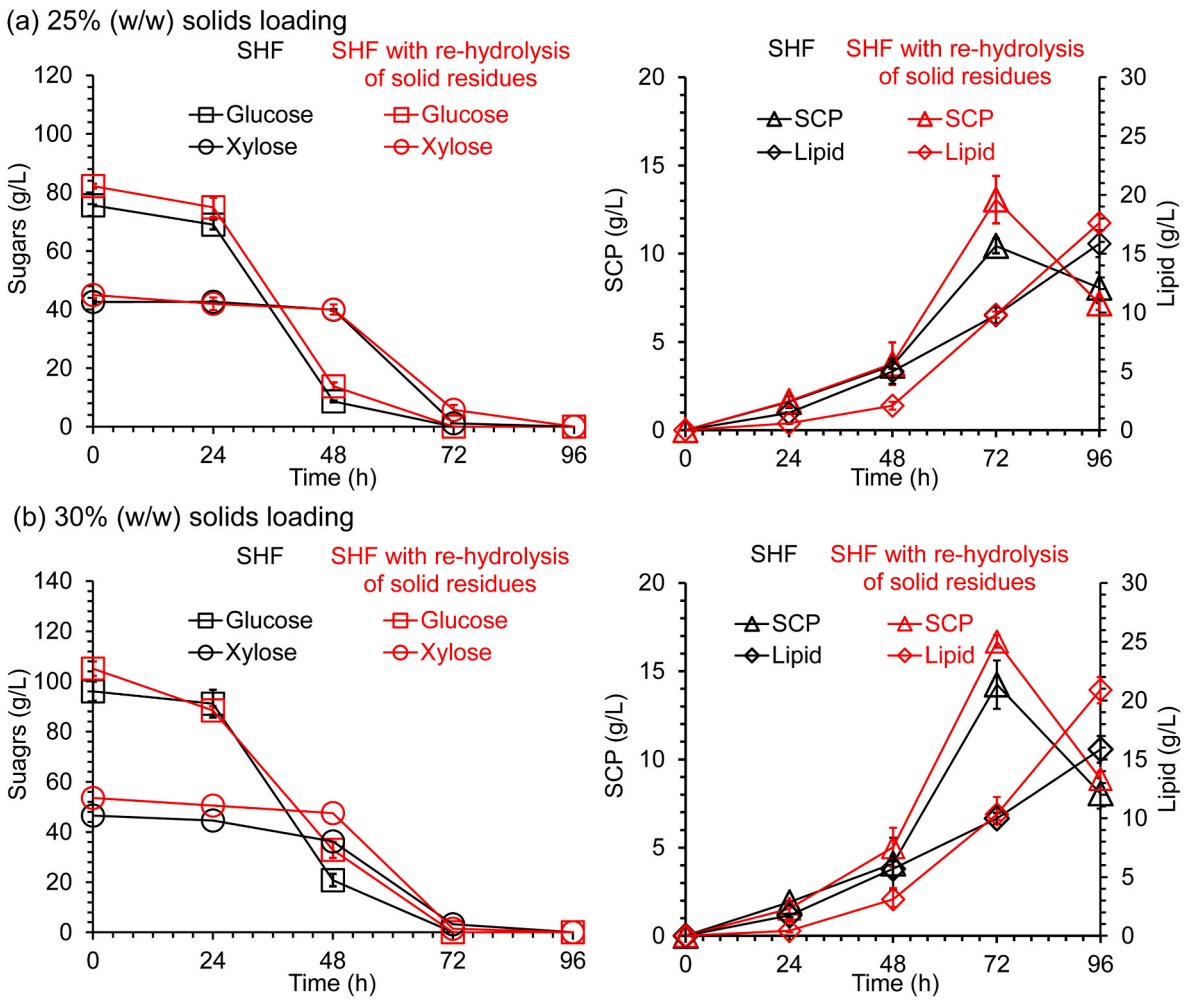

Fig. 5. Coproduction of single-cell protein (SCP) and lipid production using clarified wheat straw hydrolysate at a high concentration of nitrogen source. (a) 25 % (w/w) solids loading; (b) 30 % (w/w) solids loading. Conditions: 10 % (v/v) inoculation ratio, 30 °C, 600 rpm, 1.0 vvm, 22 g/L ammonium sulfate.

of solid residues was recycled as the process water in the next round of saccharification, ensuring the sustainable operation of the overall biorefining process. The detailed streams are shown in Fig. S3b (see supplementary materials). The results suggested that the re-hydrolysis step achieved the hydrolysis of 49.5 % of the residual cellulose. The hydrolysate derived from solid residues contained 5.09 kg of glucose and 2.34 kg of xylose, which was recovered for the next round of saccharification process water. The next round of hydrolysate contained 22.64 kg of glucose and 11.65 kg of xylose, which increased by 6.7 % and 10.0 % compared to the general saccharification. After the biodetoxification and fermentation, 5.88 kg of lipid was produced under nitrogen-limited conditions, with a 24.6 % increase compared to that in general SHF process. While 3.72 kg of single-cell proteins and 2.31 kg of lipid were coproduced under nitrogen-excessive conditions, with a 15.2 % increase in single-cell protein production compared to that in general SHF process. In summary, the overall mass balance further demonstrated that the re-hydrolysis of solid residues effectively increased the hydrolysis and fermentation yields in lignocellulosic biorefining process. This strategy is particularly relevant for processes targeting high substrate concentrations (>30 %, w/w) where product inhibition is the major bottleneck to achieving high sugar titers and yields. A detailed techno-economic analysis is crucial for assessing the commercial feasibility of this strategy, to determine if the added value (increased sugar yield, higher final sugar titer leading to lower downstream costs) can offset the added cost of the second cycle (including capital investment for larger equipment, energy for mixing). However, our present study was not designed to provide a definitive economic conclusion but rather to establish a lab-scale proof-of-concept, thereby providing a solid foundation for future research and development in lignocellulosic bioprocessing.

4. Conclusions

The re-hydrolysis of solid residues without adding fresh cellulase achieved the hydrolysis of ~50 % (w/w) of residual cellulose to glucose. The solid residues hydrolysate supernatant was recycled for the next round of saccharification. The total sugar concentration and glucose yield were increased by 9.8 % and 8.0 %, respectively, compared to the general enzymatic hydrolysis process. After the biodetoxification and fermentation, the lipid and single-cell protein titers were increased by 17.6 % and 21.0 % compared to the general SHF process. This study improved the fermentation efficiency of SHF process by re-hydrolysis of solid residues, which facilitates the efficient production of intracellular bioproducts from lignocellulose.

CRediT authorship contribution statement

Shinan Wu: Methodology, Investigation. **Zhibin Li:** Resources, Methodology. **Bin Zhang:** Writing – original draft, Supervision, Formal analysis. **Jie Bao:** Writing – review & editing, Supervision, Funding acquisition.

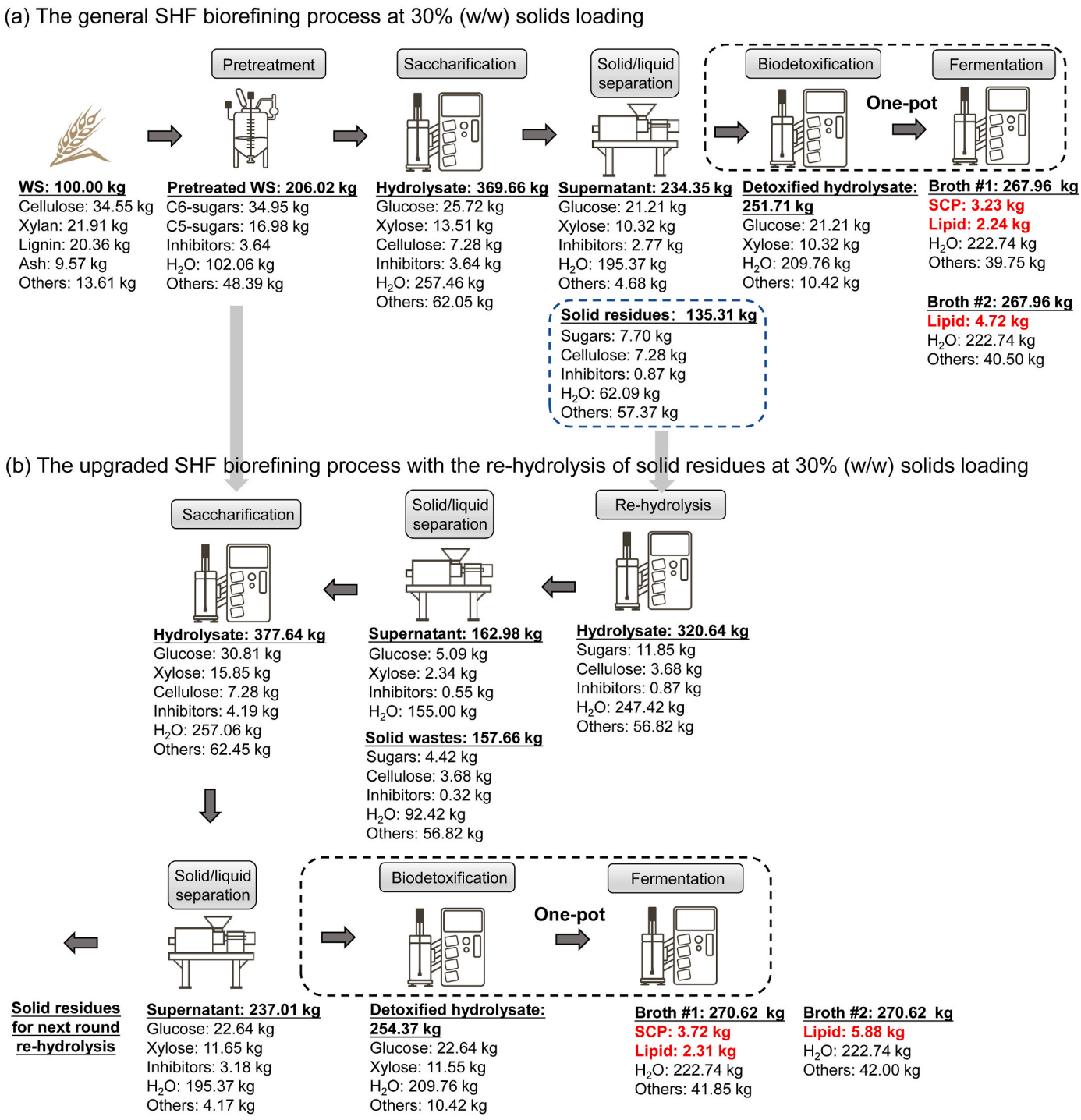

Fig. 6. Overall mass balance of dry biorefining process. (a) General separate hydrolysis and fermentation (SHF) biorefining process; (b) modified separate hydrolysis and fermentation biorefining process with the re-hydrolysis of solid residues.

Declaration of competing of interest

The authors declare that they have no competing interests.

Acknowledgments

This research was supported by the National Key R&D Program of China (2023YFA0914400), the National Natural Science Foundation of China (32301269), the Yangfan Project of Science and Technology Committee of Shanghai Municipality (23YF1409900), and the Key Technology R&D Program of the Science and Technology Commission of Shanghai Municipality (25HC2820200).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biombioe.2025.108514.

Data availability

Data will be made available on request.

References

[1] L. Zhao, Z.F. Sun, C.C. Zhang, J. Nan, N.Q. Ren, D.J. Lee, C. Chen, Advances in pretreatment of lignocellulosic biomass for bioenergy production: challenges and perspectives, Bioresour. Technol. 343 (2022) 126123, https://doi.org/10.1016/j.biortech.2021.126123.

[2] C. Cai, C. Zhang, N. Li, H. Liu, J. Xie, H. Lou, X. Pan, J.Y. Zhu, F. Wang, Changing the role of lignin in enzymatic hydrolysis for a sustainable and efficient sugar platform, Renew. Sustain. Energy Rev. 183 (2023) 113445, https://doi.org/10.1016/j.rser.2023.113445.

[3] H. Guo, Y. Zhao, J.S. Chang, D.J. Lee, Enzymes and enzymatic mechanisms in enzymatic degradation of lignocellulosic biomass: a mini-review, Bioresour. Technol. 367 (2023) 128252, https://doi.org/10.1016/j.biortech.2022.128252.

[4] P. Portero Barahona, B. Bastidas Mayorga, J. Martín-Gil, P. Martín-Ramos, E. J. Carvajal Barriga, Cellulosic ethanol: improving cost efficiency by coupling semi-continuous fermentation and simultaneous saccharification strategies, Processes 8 (11) (2020) 1459, https://doi.org/10.3390/pr8111459.

[5] G. Lamichhane, A. Acharya, D.K. Poudel, B. Aryal, N. Gyawali, P. Niraula, S. R. Phuyal, P. Budhathoki, G. Bk, N. Parajuli, Recent advances in bioethanol production from lignocellulosic biomass, Int. J. Green Energy 18 (7) (2021) 731–744, https://doi.org/10.1080/15435075.2021.1880910.

[6] R.S. Singh, T. Singh, M. Hassan, C. Larroche, Biofuels from inulin-rich feedstocks: a comprehensive review, Bioresour. Technol. 346 (2022) 126606, https://doi.org/10.1016/j.biortech.2021.126606.

[7] S. Bertacchi, P. Jayaprakash, J.P. Morrissey, P. Branduardi, Interdependence between lignocellulosic biomasses, enzymatic hydrolysis and yeast cell factories in biorefineries, Microb. Biotechnol. 15 (3) (2021) 985–995, https://doi.org/10.1111/1751-7915.13886.

[8] C.G. Liu, Y. Xiao, X.X. Xia, X.Q. Zhao, L. Peng, P. Srinophakun, F.W. Bai, Cellulosic ethanol production: progress, challenges and strategies for solutions, Biotechnol. Adv. 37 (3) (2019) 491–504, https://doi.org/10.1016/j.biotechadv.2019.03.002.

[9] S. Al-Mardeai, E. Elnajjar, R. Hashaikeh, B. Kruczek, B. Van der Bruggen, S. Al-Zuhair, Membrane bioreactors: a promising approach to enhanced enzymatic hydrolysis of cellulose, Catalysts 12 (10) (2022) 1121, https://doi.org/10.3390/catal12101121.

[10] M. Li, L. Wang, Q. Zhao, H. Chen, High concentration of fermentable sugars prepared from steam exploded lignocellulose in periodic peristalsis integrated fed-batch enzymatic hydrolysis, Appl. Biochem. Biotechnol. 194 (11) (2022) 5255–5273, https://doi.org/10.1007/s12010-022-03969-7.

[11] X. Shi, L. Wang, L. Sun, H. Chen, Sufficient premixing enhances enzymatic hydrolysis efficiency of lignocellulose at high-solids loading, Chem. Eng. J. 444 (2022) 136612, https://doi.org/10.1016/j.cej.2022.136612.

[12] C. Cai, X. Zhan, M. Zeng, H. Lou, Y. Pang, J. Yang, D. Yang, X. Qiu, Using recyclable pH-responsive lignin amphoteric surfactant to enhance the enzymatic hydrolysis of lignocelluloses, Green Chem. 19 (22) (2017) 5479–5487, https://doi.org/10.1039/c7gc02571h.

[13] C. Cai, X. Qiu, X. Lin, H. Lou, Y. Pang, D. Yang, S. Chen, K. Cai, Improving enzymatic hydrolysis of lignocellulosic substrates with pre-hydrolysates by adding cetyltrimethylammonium bromide to neutralize lignosulfonate, Bioresour. Technol. 216 (2016) 968–975, https://doi.org/10.1016/j.biortech.2016.06.043.

[14] X. Jiang, R. Zhai, H. Li, C. Li, Q. Deng, X. Wu, M. Jin, Binary additives for in-situ mitigating the inhibitory effect of lignin-derived phenolics on enzymatic hydrolysis of lignocellulose: enhanced performance and synergistic mechanism, Energy 282 (2023) 128062, https://doi.org/10.1016/j.energy.2023.128062.

[15] N. Weiss, J. Börjesson, L.S. Pedersen, A.S. Meyer, Enzymatic lignocellulose hydrolysis: improved cellulase productivity by insoluble solids recycling, Biotechnol. Biofuels 6 (1) (2013) 5, https://doi.org/10.1186/1754-6834-6-5.

[16] M.Ø. Haven, J. Lindedam, M.D. Jeppesen, M. Elleskov, A.C. Rodrigues, M. Gama, H. Jørgensen, C. Felby, Continuous recycling of enzymes during production of lignocellulosic bioethanol in demonstration scale, Appl. Energy 159 (2015) 188–195, https://doi.org/10.1016/j.apenergy.2015.08.062.

[17] Z. Yu, H. Jameel, H.-m. Chang, R. Philips, S. Park, Quantification of bound and free enzymes during enzymatic hydrolysis and their reactivities on cellulose and lignocellulose, Bioresour. Technol. 147 (2013) 369–377, https://doi.org/10.1016/j.biortech.2013.08.010.

[18] D. Xin, M. Yang, X. Chen, Y. Zhang, R. Wang, P. Wen, J. Zhang, Improving cellulase recycling efficiency by decreasing the inhibitory effect of unhydrolyzed solid on recycled corn stover saccharification, Renew. Energy 145 (2020) 215–221, https://doi.org/10.1016/j.renene.2019.06.029.

[19] M. Jin, L. da Costa Sousa, C. Schwartz, Y. He, C. Sarks, C. Gunawan, V. Balan, B. E. Dale, Toward lower cost cellulosic biofuel production using ammonia based pretreatment technologies, Green Chem. 18 (4) (2016) 957–966, https://doi.org/10.1039/c5gc02433a.

[20] B. Qi, X. Chen, Y. Su, Y. Wan, Enzyme adsorption and recycling during hydrolysis of wheat straw lignocellulose, Bioresour. Technol. 102 (3) (2011) 2881–2889, https://doi.org/10.1016/j.biortech.2010.10.092.

[21] W. Zheng, Y. Qin, X. Zhuang, H. Xiao, C. Liu, X. Fu, Q. Lin, T. Lan, Recycling enzymatic hydrolysis lignin residues saved cellulase in enzymatic hydrolysis of lignocellulose: an insight from cellulase adsorption mechanism, Ind. Crop. Prod. 208 (2024) 117884, https://doi.org/10.1016/j.indcrop.2023.117884.

[22] M.H. Vasconcelos, F.M. Mendes, L. Ramos, M.O.S. Dias, A. Bonomi, C.D.F. Jesus, M.D.B. Watanabe, T.L. Junqueira, A.M.F. Milagres, A. Ferraz, J.C.d. Santos, Techno-economic assessment of bioenergy and biofuel production in integrated sugarcane biorefinery: identification of technological bottlenecks and economic feasibility of dilute acid pretreatment, Energy 199 (2020) 117422, https://doi.org/10.1016/j.energy.2020.117422.

[23] A. Sluiter, B. Hames, C. Scarlata, J. Sluiter, D. Templeton, Determination of Structural Carbohydrates and Lignin in Biomass National Renewable, NREL, Golden, CO, 2012. NREL/TP-510–42618. Retrieved from, http://www.nrel.gov/docs/gen/fy08/42618.pdf.

[24] X. Han, J. Bao, General method to correct the fluctuation of acid based pretreatment efficiency of lignocellulose for highly efficient bioconversion, ACS Sustain. Chem. Eng. 6 (3) (2018) 4212–4219, https://doi.org/10.1021/acssuschemeng.7b0460.

[25] B. Zhang, D. Ren, Q. Liu, X. Liu, J. Bao, Coproduction of single-cell protein and lipid from lignocellulose derived carbohydrates and inorganic ammonia salt with soluble ammonia recycling, Bioresour. Technol. 384 (2023) 129345, https://doi.org/10.1016/j.biortech.2023.129345.

[26] Q. Liu, M. Lu, C. Jin, W. Hou, L. Zhao, J. Bao, Ultra-centrifugation force in adaptive evolution changes the cell structure of oleaginous yeast *Trichosporon cutaneum* into a favorable space for lipid accumulation, Biotechnol. Bioeng. 119 (6) (2022) 1509–1521, https://doi.org/10.1002/bit.28060.

[27] T. Han, B. Zhang, H. Yang, X. Liu, J. Bao, Changes in pH values allow for a visible detection of the end point in submerged liquid biodetoxification during biorefinery processing, ACS Sustain. Chem. Eng. 11 (46) (2023) 16608–16617, https://doi.org/10.1021/acssuschemeng.3c04986.

[28] B. Zhang, F.A. Khushik, B. Zhan, J. Bao, Transformation of lignocellulose to starch-like carbohydrates by organic acid-catalyzed pretreatment and biological detoxification, Biotechnol. Bioeng. 118 (10) (2021) 4105–4118, https://doi.org/10.1002/bit.27887.

[29] W. Ying, J. Zhu, Y. Xu, J. Zhang, High solid loading enzymatic hydrolysis of acetic acid-peroxide/acetic acid pretreated poplar and cellulase recycling, Bioresour. Technol. 340 (2021) 125624, https://doi.org/10.1016/j.biortech.2021.125624.

[30] C. Xu, J. Zhang, Y. Zhang, Y. Guo, H. Xu, J. Xu, Z. Wang, Enhancement of high-solids enzymatic hydrolysis efficiency of alkali pretreated sugarcane bagasse at low cellulase dosage by fed-batch strategy based on optimized accessory enzymes and additives, Bioresour. Technol. 292 (2019) 121993, https://doi.org/10.1016/j.biortech.2019.121993.

[31] G. Liu, Q. Zhang, H. Li, A.S. Qureshi, J. Zhang, X. Bao, J. Bao, Dry biorefining maximizes the potentials of simultaneous saccharification and co-fermentation for cellulosic ethanol production, Biotechnol. Bioeng. 115 (1) (2018) 60–69, https://doi.org/10.1002/bit.26444.

[32] J. Zhang, D. Chu, J. Huang, Z. Yu, G. Dai, J. Bao, Simultaneous saccharification and ethanol fermentation at high corn stover solids loading in a helical stirring bioreactor, Biotechnol. Bioeng. 105 (4) (2009) 718–728, https://doi.org/10.1002/bit.22593.

[33] S.N. Olsen, K. Borch, N. Cruys-Bagger, P. Westh, The role of product inhibition as a yield-determining factor in enzymatic high-solid hydrolysis of pretreated corn stover, Appl. Biochem. Biotechnol. 174 (1) (2014) 146–155, https://doi.org/10.1007/s12010-014-1049-1.

[34] X. Jiang, R. Zhai, M. Jin, Increased mixing intensity is not necessary for more efficient cellulose hydrolysis at high solid loading, Bioresour. Technol. 329 (2021) 124911, https://doi.org/10.1016/j.biortech.2021.124911.

[35] S.K. Paul, S. Chakraborty, Mixing effects on the kinetics of enzymatic hydrolysis of lignocellulosic *Sunn hemp* fibres for bioethanol production, Chem. Eng. J. 377 (2019) 120103, https://doi.org/10.1016/j.cej.2018.10.040.

[36] D. Humbird, R. Davis, L. Tao, C. Kinchin, D. Hsu, A. Aden, P. Schoen, J. Lukas, B. Olthof, M. Worley, D. Sexton, D. Dudgeon, Process Design and Economics for Biochemical Conversion of Lignocellulosic Biomass to Ethanol: Dilute-Acid Pretreatment and Enzymatic Hydrolysis of Corn Stover, NREL, Golden, CO, 2011, https://doi.org/10.2172/1013269. NREL/TP-5100-47764.

[37] B. Zhang, Q. Han, Q. Liu, Y. Wang, J. Bao, Recycling of waste calcium carbonate in lignocellulosic biorefining chain for chiral lactic acid production, Bioresour. Technol. 395 (2024) 130303, https://doi.org/10.1016/j.biortech.2024.130303.

[38] H. Guo, Y. Zhao, J.S. Chang, D.J. Lee, Inhibitor formation and detoxification during lignocellulose biorefinery: a review, Bioresour. Technol. 361 (2022) 127666, https://doi.org/10.1016/j.biortech.2022.127666.

[39] H. Ran, J. Zhang, Q. Gao, Z. Lin, J. Bao, Analysis of biodegradation performance of furfural and 5-hydroxymethylfurfural by *Amorphotheca resinae* ZN1, Biotechnol. Biofuels 7 (1) (2014) 51, https://doi.org/10.1186/1754-6834-7-51.

[40] X. Yi, Q. Gao, L. Zhang, X. Wang, Y. He, F. Hu, J. Zhang, G. Zou, S. Yang, Z. Zhou, J. Bao, Heterozygous diploid structure of *Amorphotheca resinae* ZN1 contributes efficient biodetoxification on solid pretreated corn stover, Biotechnol. Biofuels 12 (2019) 126, https://doi.org/10.1186/s13068-019-1466-z.

[41] B. Zhang, S. Wu, A. Chen, J. Bao, Harnessing *Paecilomyces variotii* to remove lignocellulose-derived inhibitors: potential evaluation, lignocellulosic biorefining route design, and transcriptome analysis, Syst. Microbiol. Biomanuf. (2025), https://doi.org/10.1007/s43393-025-00393-w.

[42] Y. Zhang, J. Bao, Tolerance of T*richosporon cutaneum* to lignin derived phenolic aldehydes facilitate the cell growth and cellulosic lipid accumulation, J. Biotechnol. 343 (2022) 32–37, https://doi.org/10.1016/j.jbiotec.2021.09.009.

[43] B. Bharathiraja, S. Sridharan, V. Sowmya, D. Yuvaraj, R. Praveenkumar, Microbial oil - a plausible alternate resource for food and fuel application, Bioresour. Technol. 233 (2017) 423–432, https://doi.org/10.1016/j.biortech.2017.03.006.

[44] L. Chen, X. Qian, X. Zhang, X. Zhou, J. Zhou, W. Dong, F. Xin, W. Zhang, M. Jiang, K. Ochsenreither, Co-production of microbial lipids with valuable chemicals, Biofuel Bioprod. Biorefining 15 (3) (2021) 945–954, https://doi.org/10.1002/bbb.2209.

[45] M. Sharif, M.H. Zafar, A.I. Aqib, M. Saeed, M.R. Farag, M. Alagawany, Single-cell protein: sources, mechanism of production, nutritional value and its uses in aquaculture nutrition, Aquaculture 531 (2021) 735885, https://doi.org/10.1016/j.aquaculture.2020.735885.

[46] H. Yu, L. Shan, L. Li, Q. Zhang, D. Liu, Effect of dietary lipid levels on the antioxidant responses, initial immunity, and mTOR signaling in the liver of coho salmon (*Oncorhynchus kisutch*), Aquaculture Reports 23 (2022) 101090, https://doi.org/10.1016/j.aqrep.2022.101090.

[47] D. Kumar, B. Singh, J. Korstad, Utilization of lignocellulosic biomass by oleaginous yeast and bacteria for production of biodiesel and renewable diesel, Renew. Sustain. Energy Rev. 73 (2017) 654–671, https://doi.org/10.1016/j.rser.2017.01.022.

[48] P. Thiviya, A. Gamage, R. Kapilan, O. Merah, T. Madhujith, Production of single-cell protein from fruit peel wastes using *Palmyrah toddy* yeast, Fermentation 8 (8) (2022) 355, https://doi.org/10.3390/fermentation8080355.