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RESEARCH ARTICLE

High-solids enzymatic saccharification of starch-rich raw herbal biomass residues for producing high titers of glucose

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Abstract

The bioresource utilization of herbal biomass residues (HBRs) has been receiving more attention. Herein, three different HBRs from *Isatidis Radix* (IR) and *Sophorae Flavescentis Radix* (SFR) and *Ginseng Radix* (GR) were subjected to batch and fed-batch enzymatic hydrolysis to produce high-concentration glucose. Compositional analysis showed the three HBRs had substantial starch content (26.36–63.29%) and relatively low cellulose contents (7.85–21.02%). Due to their high starch content, the combined action of cellulolytic and amylolytic enzymes resulted in greater release of glucose from the raw HBRs compared to using the individual enzyme alone. Batch enzymatic hydrolysis of 10% (w/v) raw HBRs with low loadings of cellulase (≤ 10 FPU/g substrate) and amylolytic enzymes (≤ 5.0 mg/g substrate) led to a high glucan conversion of ≥ 70%. The addition of PEG 6000 and Tween 20 did not contribute to glucose production. Furthermore, to achieve higher glucose concentrations, fed-batch enzymatic hydrolysis was conducted using a total solid loading of 30% (w/v). After 48-h of hydrolysis, glucose concentrations of 125 g/L and 92 g/L were obtained for IR and SFR residues, respectively. GR residue yielded an 83 g/L glucose concentration after 96 h of digestion. The high glucose concentrations produced from these raw HBRs indicate their potential as ideal substrate for a profitable biorefinery. Notably, the obvious advantage of using these HBRs is the elimination of the pretreatment step, which is typically required for agricultural and woody biomass in similar studies.

Keywords Enzymatic hydrolysis · Cellulase · Amylolytic enzymes · Chinese herbal residues · Fed-batch · Non-ionic surfactants

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Introduction

Renewable lignocellulosic biomass is widely recognized as an abundant and cost-effective feedstock that can replace non-renewable fossil resources, such as coal, petroleum, and natural gas in the production of transportation fuel, functional materials, and valuable chemicals (Gupta et al. 2022b). However, its considerable recalcitrance to biocatalysts poses a significant challenge in achieving efficient and economically viable bioconversion of cellulosic feedstock (Himmel et al. 2007). To address this challenge, pretreatment is widely acknowledged as a prerequisite step to achieve high-yield bioconversion of carbohydrate polymers in lignocellulosic biomass (Gupta et al. 2022a).

Typically, biomass pretreatments involve the use of chemicals at high temperatures and/or high pressures to break down the rigid lignocellulosic structures. However, these pretreatment methods are energy-intensive, economically costly, and environmentally unfriendly. Consequently, using non-pretreated raw feedstock and avoiding pretreatment step

in biomass bioconversion can remarkably increase the economic feasibility of the process. However, to the best of our knowledge, there are currently no reports available on highly efficient enzymatic hydrolysis of non-pretreated agricultural crop residues and forest woody residues (Carpenter et al. 2014).

Herbal biomass residues (HBRs) refer to the residual material left after the feedstock processing and extraction of active ingredients from medicinal herbs. Unfortunately, they have received less attention compared to agricultural and woody residues (Huang et al. 2021; Lu and Li 2021; Tao et al. 2021). Currently, China's annual discharge of HBRs has increased exponentially to 60–70 million tons (Lu and Li 2021). Alike to lignocellulosic materials, HBRs mainly consist of cellulose, hemicellulose and lignin, making them suitable feedstock for biorefinery application. Nevertheless, only a few HBRs have been investigated for their bioconversion potential, and all these studies entailed pretreatment prior to bioconversion, as summarized in recent reviews (Huang et al. 2021; Lu and Li 2021; Tao et al. 2021). In our recent work, we made a significant breakthrough by demonstrating that four raw HBRs obtained after the decoction of *Glycyrrhizae Radix et Rhizoma*, *Radix Astragali*, *Magnoliae Officinalis Cortex*, and *Scutellariae Radix* can be directly and efficiently hydrolyzed using combined cellulase and amylolytic enzymes (Zhu et al. 2022). The finding was based on the identification of starch in addition to cellulose in these HBRs, which was overlooked by previous researchers due to the use of the two-step acid hydrolysis protocol developed by the US National Renewable Energy Laboratory (NREL) to for compositional analysis of HBRs. Unfortunately, the NREL procedure cannot distinguish between glucose derived from starch and glucose derived from cellulose after acidic hydrolysis of the two polysaccharides (Sluiter et al. 2021).

Based on our previous study, we hypothesized that direct hydrolysis strategy using combined cellulolytic and amylolytic enzymes could be suitable for raw HBRs containing both starch and cellulose. To validate this hypothesis, we analyzed the chemical compositions of seven HBRs derived from commonly used traditional Chinese medicine and found that all of them contain both starch and cellulose (see Table S1 in the Supplementary Material). Notably, the residues of *Isatidis Radix* (IR, Banlangen in Chinese), *Sophorae Flavescentis Radix* (SFR, Kushen in Chinese), and *Ginseng Radix* (GR, Renshen in Chinese) exhibited a glucan (cellulose and starch) composition of ≥ 39% with starch accounting for over half of the glucan content. These three HBRs appear to be highly suitable biomass feedstock to produce high-concentration glucose syrup through high-solids enzymatic hydrolysis. Microbial fermentation of the high-concentration glucose feed leads to production of a variety of bio-products with high concentration, significantly improving the profitability of HBRs biorefinery by reducing the capital investment and operational costs. However, high-solids enzymatic hydrolysis of lignocellulosic biomass, typically with substrate concentration exceeding 15%, encounters the challenge related to mixing, which can lead to reduced mass and heat transfer efficiency. Therefore, this can considerably decrease the sugar yield and increase the energy consumption (Shiva et al. 2022). To overcome these challenges, the fed-batch strategy has been proven to be very effective in high-solids enzymatic hydrolysis by gradually feeding the substrate into the reactor multiple times instead of a one-time addition. This approach helps maintain a low viscosity in the hydrolysis system, addressing the mixing issues associated with high solid loading (Battista and Bolzonella 2018). Considering that starch is enzymatically digested within several hours compared to several days typically required for enzymatic cellulose hydrolysis (Zhu et al. 2022), the high-solids effect during enzymatic conversion of HBRs is expected to be less pronounced compared to conventional lignocellulosic biomass, such as agricultural and woody biomass, which does not contain starch.

The objective of this study was to assess the feasibility of utilizing three raw non-pretreated HBRs with high starch contents, namely residues of IR, SFR, and GR, as feedstock for direct enzymatic production of high-concentration glucose employing combined cellulolytic and amylolytic enzymes. The influences of substrate concentration, enzyme dosage and addition order of enzymes, and non-ionic surfactants on the batch enzymatic saccharification of these raw HBRs were investigated to maximize the glucan hydrolysis. Furthermore, high-solids enzymatic hydrolysis of the three raw HBRs using the fed-batch strategy was conducted to attain a hydrolysate with a high glucose concentration, essential for economically viable production of bio-based products. This study offers valuable insights into the efficient and cost-effective utilization of starch-rich HBRs.

Materials and methods

Materials

The three starch-rich HBRs were obtained from Institute of Chinese Materia Medica, Chinese Academy of Chinese Medical Sciences. They were prepared by decoction of IR, SFR and GR, respectively, and then dried at 60 °C until reaching a constant weight for further use. Two non-ionic surfactants, i.e., polyethylene glycol (PEG) 6000 and Tween 80 purchase from Xilong Scientific Co., Ltd. (Guangdong, China) and were of chemically pure grade. The source, protein content, activity of cellulase, α-amylase, and amyloglucosidase used in this study were detailed in our previous

work (Zhu et al. 2022). Antibiotic antimycotic solution (A5955) was acquired from Sigma-Aldrich, Shanghai, China.

Batch and fed-batch enzymatic hydrolysis

Typically, batch enzymatic saccharification of the raw HBRs was carried out in a 50 mM sodium citrate buffer (pH 4.8) at a substrate concentration of 10% (w/v, dry weight basis). To prevent microbial contamination, an antibiotic antimycotic solution was added at a concentration of 1% (v/v). Different loadings of cellulase and amylolytic enzymes (an equal-volume combination of α-amylase and amyloglucosidase) ranging from 5 to 40 FPU/g substrate and 0.05 to 5 mg protein/g substrate, respectively, were added separately or in combination to the Erlenmeyer flask to starch the saccharification process. The effect of non-ionic surfactants was investigated by supplementing different concentrations of PEG 6000 and Tween 80 ranging from 20 to 60 mg/g substrate into the reaction system. The one-time-at-a-factor method was used to evaluate the effects of these factors and determine the operating conditions, which that were subsequently used in the fed-batch enzymatic hydrolysis.

For the fed-batch enzymatic hydrolysis at a final high-solid loading of 30% (w/v), the saccharification experiment was initiated at a solid loading of 10% or 15% (w/v). The remaining 20% or 15% solid was added twice (at 4 and 8 h) or three times (at 3, 6, and 9 h) to the flask. The cellulolytic and amylolytic enzymes were introduced together at the onset of the hydrolysis reaction.

Both batch and fed-batch enzymatic hydrolysis experiments were performed on a rotary shaker at 50 °C and 150 rpm. Samples were collected at different time intervals and boiled to deactivate the enzymes. The glucose concentration in the hydrolysate samples was determined using high-performance liquid chromatography (HPLC). The glucan conversion of the HBRs through enzymatic hydrolysis of glucan component (cellulose and starch) using combined enzymes was calculated using the following equation:

$$Glucan\ conversion(\%) = \frac{Glucose\ from\ enzymatic\ hydrolysis\ of\ glucan(g)}{Glucan\ content\ in\ HBR(g) \times 1.11} \times 100 \quad (1)$$

Similarly, the starch conversion of the HBRs through enzymatic digestion of starch using only amylolytic enzymes (without cellulose hydrolysis capabilities) was determined using the following equation:

$$Starch\ conversion(\%) = \frac{Glucose\ from\ enzyamtic\ hydrolysis\ of\ starch\ (g)}{Starch\ in\ HBR(g) \times 1.11} \times 100 \quad (2)$$

It was worthwhile mentioning that a range of reference blank controls that consisted of all reaction components except for the HBRs were also performed to account for any glucose contribution from the enzyme solution itself. To ensure accurate calculation of the glucan and starch conversions, the glucose content resulting from the addition of enzymes, either individually or in combination, was subtracted from each sample before calculating the conversions.

Analytical methods

The main compositions of HBR, namely total glucan, hemicellulose, and lignin were analyzed using the method outlined by Sluiter et al. (2008). The content of cellulosic glucan was measured following the procedure described by Sluiter et al. (2021). Briefly, the starch-containing biomass is first treated with amylolytic enzymes to remove starch followed by a two-stage acid hydrolysis to determine the cellulose content of the biomass. The starch content in the HBR samples was measured using the rapid total starch-NaOH procedure reported by McCleary et al. (2019). The quantification of monomeric sugars, such as glucose and xylose, was conducted following a previously established method described elsewhere (Li et al. 2020).

The FPA, xylanase activity and β-glucosidase activity of the cellulase preparation were assayed following the procedures outlined by Ghose (1987), Bailey et al. (1992) and Bailey and Nevalainen (1981), respectively. The activities of amylolytic enzymes were measured according to the methods described by Leaes et al. (2013). The protein concentrations of these enzymes were quantified following the ninhydrin assay method with alkaline hydrolysis (Haven and Jørgensen 2014).

Results and discussion

The main chemical compositions of the HBRs

Table 1 presents the chemical compositions of the three raw HBRs. The results revealed substantial total glucan content, accounting for around 70%, 50%, and 40% of the residues of IR, SFR, and GR, respectively. The total glucan content was determined using the two-step acid hydrolysis method recommend by US NREL (Sluiter et al. 2008). Interestingly, the total glucan content closely matched the combined content of starch glucan and cellulosic glucan, indicating that the constituents of total glucan in these three HBRs are primarily starch and cellulose. Among the three HBRs, IR residue contained the highest starch content (63.29%) but the lowest cellulose content (7.85%). On the other hand, both SFR (29.71%) and GR (26.36%) demonstrated comparable starch content, but SFR residue had a significantly higher cellulose content (21.02%) compared to GR residue (12.67%).

Table 1 The main chemical compositions of the three HBRs (% on dry weight basis)

CMHRs	Total glucan	Starch glucan	Cellulosic glucan	Xylan	Arabinan	Lignin
IR	69.89 ± 1.22	63.29 ± 0.42	7.85 ± 0.13	5.19 ± 0.04	3.83 ± 0.03	11.68 ± 0.67
SFR	49.21 ± 0.44	29.71 ± 0.87	21.02 ± 0.16	6.53 ± 0.12	5.15 ± 0.13	9.89 ± 0.32
GR	39.21 ± 0.26	26.36 ± 0.55	12.67 ± 0.34	8.12 ± 0.08	5.93 ± 0.07	6.69 ± 0.26

IR, SFR, and GR stand for *Isatidis Radix* (Banlangen in Chinese), *Sophorae Flavescentis Radix* (Kushen in Chinese) and *Ginseng Radix* (Renshen in Chinese), respectively

Additionally, small amounts of hemicellulose were detected in all three HBRs. The content of xylan varied between 5.19 and 8.12%, while the content of arabinan fluctuated narrowly between 3.83 and 5.93%. The lignin contents of the three HBRs ranged from 6.69 to 11.68%. It was worth noting that the lignin content in the herbal residue was determined according to the method commonly used for the analysis of conventional lignocellulosic biomass. As herbal residue as a non-conventional biomass, it is possible that certain components resistant to acid hydrolysis used in biomass composition analysis may be present. Consequently, there is a probability of overestimated lignin content in Table 1. Nevertheless, the lignin contents of the three HBRs were significantly lower than those typically found in commonly investigated crop and woody biomass residues (Saravanan et al. 2022). The high glucan contents coupled with the low lignin content of these three HBRs highlight their promising potential as feedstock for bioconversion processes aimed at producing value-added products.

Hydrolysis of the HBRs with combined cellulolytic and amylolytic enzymes

The compositional analysis of the HBRs revealed that all the three HBRs contain varying percentages of starch and cellulose (as shown in Table 1). It was expected that glucose production from hydrolysis of these HBRs would be maximized by employing a blend of cellulase and amylolytic enzymes. To explore the potential synergistic effect, the HBRs were hydrolyzed using a combination of both enzymes at a solid loading of 10% (w/v), with a single enzyme serving as a control. The results depicted in Fig. 1a and b demonstrated that the combined use of both enzymes resulted in significantly higher glucose production and glucan conversion compared to their separate usage. For instance, the 72-h glucan conversions of the IR, SFR, and GR residues with combined enzymes were 70.00%, 71.93%, and 79.38% (Fig. 1b), respectively. The glucan conversions at 72 h showed 1.16-, 1.59-, and 1.47-fold increases for the IR, SFR, and GR residues, respectively, when compared to hydrolysis with individual amylolytic enzymes. Specifically, the IR residue demonstrated a smaller increment in glucan conversion, likely due to its high starch content, which can be readily digested by amylolytic enzymes.

In contrast, hydrolysis with only cellulase resulted in 72-h glucan conversions of approximately 49%, 61%, and 64% for the IR, SFR, and GR residues, respectively (Fig. 1b). These glucan conversion data would not be achieved if only cellulose fraction was hydrolyzed by cellulase, as the cellulose content accounted for only about 11%, 43%, and 32% of total glucan in the IR, SFR, and GR residues, respectively, as calculated from Table 1. Our previous investigation indicated that amylolytic enzymes used in the present study cannot hydrolyze cellulose into glucose, but the cellulase blend demonstrated certain ability to depolymerize starch into glucose (Zhu et al. 2022). Therefore, the amylolytic activities present in the cellulase cocktail are likely responsible for the high glucan conversion observed when using cellulase alone for hydrolysis of the three HBRs. Comparatively, the combination of cellulase and amylolytic enzymes led to 1.43-, 1.18-, and 1.24-fold increases in 72-h glucan conversions for the IR, SFR, and GR residues, respectively. Despite the SFR residue having the highest cellulose content among the three HBRs, it exhibited the lowest increase in glucan conversion when combined cellulolytic and amylolytic enzymes were used. This observation suggests that the SFR residue was most resistant to hydrolysis using cellulase, indicating a higher degree of recalcitrance compared to the other HBRs. The remarkable enhancement in glucan conversions observed with the combined enzymes, as compared to the use of an individual enzyme, can be due to the synergistic depolymerization of starch and cellulose into glucose. Additionally, it has been reported that the incorporation of cellulase along with amylolytic enzymes containing α-amylase, when hydrolyzing starch-containing lignocellulosic feedstock can alleviate the inhibitory effect of cellulose on α-amylase, thereby promoting starch digestion by amylolytic enzymes (Shokrkar and Ebrahimi 2018).

It is worth noting that the evaluation and comparison of the performance of separate and combined enzymes was based on glucan conversion, which includes the conversion of both starch and cellulose. The amylolytic enzymes used in the present study exhibit exceptional specificity, solely catalyzing the conversion of starch into glucose. Thus, when hydrolysis was performed using only amylolytic enzymes,

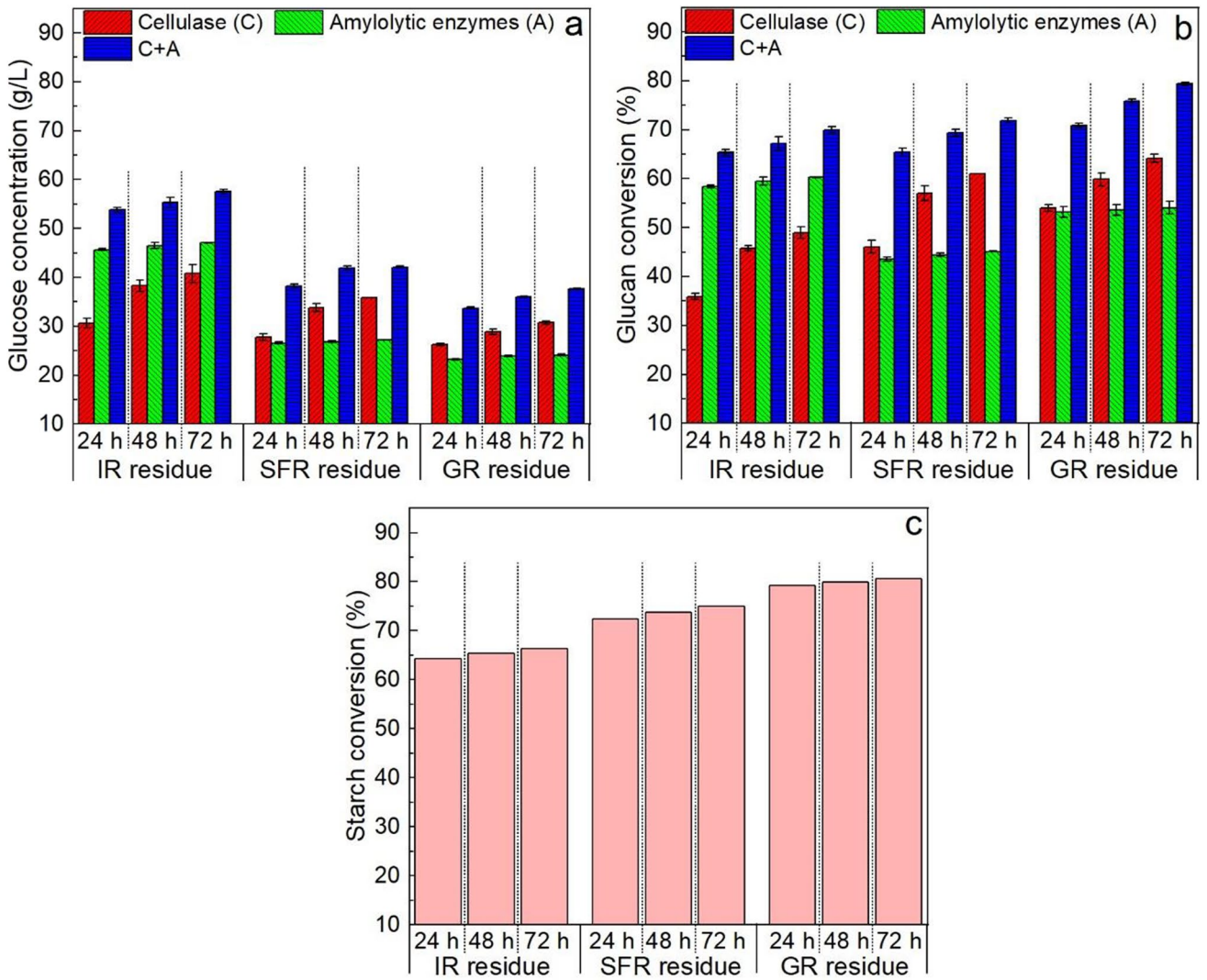

Fig. 1 Effect of separate and combined cellulase and amylolytic enzymes on glucose concentration (**a**) and glucan conversion (**b**) during the hydrolysis of the three HBRs and the changing trend of starch conversion (**c**) during the hydrolysis of the HBRs with amylolytic enzymes alone. The HBRs were hydrolyzed at 10% (w/v) solids loading using a combination of 10 FPU/g substrate of cellulase and 5 mg/g substrate of amylolytic enzymes

starch conversion was calculated for the three HBRs. As depicted in Fig. 1c, the 72-h starch conversions for the IR, GFR, and SR residues were 66.31%, 74.93%, and 80.55%, respectively. The lowest starch conversion observed in the IR residue was likely attributed to its more recalcitrant structure than the other two HBRs. Moreover, the inhibitory effect of glucose as an end-product may have contributed to the reduced glucan conversion. This can be observed from Fig. 1a, where hydrolysis of the IR residue with individual amylolytic enzymes resulted in a glucose concentration of 47 g/L at 72 h, which exceeds the threshold concentration known to inhibit amylase (Baks et al. 2006; Reddy and Abouzied 1986). Furthermore, Fig. 1a indicates that when individual amylolytic enzymes were used for hydrolysis, a high level of glucan conversion was achieved at 24 h and remained unchanged when the reaction time was extended to 72 h. In contrast, when hydrolysis was performed using cellulase alone, the glucan conversion continued to increase from 24 to 72 h. Although the starch content in these HBRs is higher than the cellulose content, the starch fraction is hydrolyzed at a much faster rate than cellulose. This observation could be attributed to two factors. On the one hand, starch is markedly more easily hydrolyzed by amylolytic enzymes compared to cellulose hydrolysis by cellulase. It has been reported that the hydrolysis rate of starch was 100 times faster than of cellulose (Banerjee et al. 2010). On the other hand, the starch located inside plant cell is likely to be more accessible than cellulose, which interacts with hemicellulose and lignin to form the primary component of the plant cell wall. The presence of hemicellulose and lignin

in the cell wall can create a physical barrier that limits the accessibility of cellulose to cellulase.

In our previous study, hydrolysis of three low-starch HBRs with combined enzymes resulted in 48-h glucan conversion rates ranging from 62 to 69% at a substrate loading of 2% (w/v) (Zhu et al. 2022). In the present study, hydrolysis of three high-starch HBRs was investigated at a higher substrate loading of 10% (w/v). Remarkably, the 48-h glucan conversion rates achieved by high-starch HBRs were comparable to those obtained from the hydrolysis of the low-starch HBRs. This suggests that despite the higher starch content, the high-starch HBRs exhibit similar hydrolysis efficiency to the low-starch HBRs, even at a higher substrate loading. These results highlight the potential of high-starch HBRs as promising feedstocks for bioconversion processes.

The effect of solid loading on glucan conversion in the HBRs

Solid loading plays an important role in enzymatic saccharification of lignocellulosic biomass. Therefore, the effect of solid loading, ranging from 5 to 15% (w/v), on enzymatic saccharification of the three HBRs was explored. The cellulase and amylolytic enzymes were simultaneously added at the beginning of the hydrolysis process. As depicted in Fig. 2a and b, regardless of the HBR used for hydrolysis, higher substrate concentration resulted in increased glucose concentration but decreased glucan conversion. For example, when the substrate concentration was increased from 5 to 15% (threefold increase), the glucose concentration in the hydrolysate after 72 h of hydrolysis of the IR, SFR, and GR residues increased by 3.00-, 2.82-, and 2.83-fold, reaching 87.63, 62.99, and 53.34 g/L, respectively (Fig. 2a). However, the corresponding glucan conversions decreased by 5.89%, 7.95%, and 6.19%, reaching 67.08%, 71.04%, and 74.61%, respectively (Fig. 2b). The nearly linear increase in glucose concentration with increasing substrate concentration from 5 to 15% (w/v) was unexpected for conventional lignocellulosic biomass, which showed linear decrease in glucan conversion with increasing solids concentration (Kristensen et al. 2009). This suggested that the tested three starch-rich HBRs were readily hydrolyzed by the combined cellulolytic and amylolytic enzymes. The positive relationship between substrate concentration and glucose concentration in the hydrolysate could be explained by the higher glucan content available for saccharification at higher substrate concentrations. However, the weak negative correlation between substrate concentration and glucan conversion observed in the this study could be caused by limited mass and heat transfer in the reactor resulting from the high viscosity of the hydrolytic slurry at high-solids loading (da Silva et al. 2020). At a low substrate concentration of 5% (w/v), which is known to mitigate mixing problem (Modenbach and Nokes 2013), the 72-h glucan conversions of the raw IR, SFR, and GR residues using the combined enzymes were 71.28%, 77.17%, and 79.53%, respectively. These high glucan conversion results cannot be achieved by enzymatic hydrolysis of raw non-pretreated conventional lignocellulosic biomass, suggesting that the three tested raw HBRs exhibited significantly lower recalcitrance towards enzymatic hydrolysis.

Additionally, it was observed that regardless of the substrate concentration used, hydrolysis of the IR residue produced the highest glucose concentration in the hydrolysate, followed by hydrolysis of the SFR residue, while GR residue

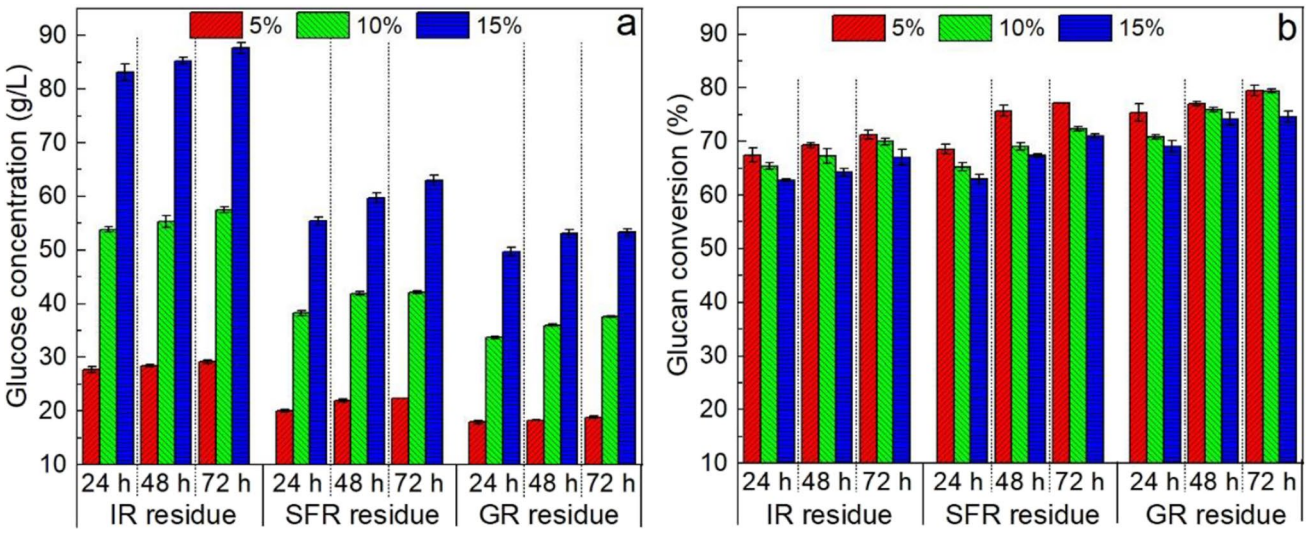

Fig. 2 Effect of solids loading on glucose concentration (**a**) and glucan conversion (**b**) during the hydrolysis of the three HBRs using a combination of 10 FPU/g substrate of cellulase and 5 mg/g substrate of amylolytic enzymes

yielded the lowest glucose release (Fig. 2a). This observation was most likely due to the significant difference in their glucan content, as indicated in Table 1. When hydrolyzed with a fixed enzyme loading at a specific substrate concentration, the amount of glucose released into the hydrolysate is expected to be positively correlated with the glucan contents of the HBRs. Furthermore, as discussed earlier, starch is more readily digestible than cellulose. Taking into the consideration of the highest starch content in IR residue, it can be concluded that the at any given hydrolysis time point, the viscosity-induced mass and heat transfer limitations were least severe in the hydrolytic slurry of the IR residue, thus promoting glucose production. On the contrary, high glucan conversion was observed in enzymatic hydrolysis of HBRs with low glucan content. Specifically, the highest glucan conversion was observed from hydrolysis of the GR residue, followed by the SFR residue, while hydrolysis of the IR residue exhibited the lowest glucose conversion (Fig. 2b). This phenomenon could be a result of the inhibitory effects of sugar products on both cellulolytic and amylolytic enzymes (Andrić et al. 2010; Baks et al. 2006; Reddy and Abouzied 1986). The higher the glucose concentration in the hydrolysate, the more pronounced the inhibition of glucose on hydrolytic enzymes, leading to reduced glucose release from glucan.

The effect of enzyme loading and enzyme addition order on glucan conversion in the HBRs

The combined addition of cellulolytic and amylolytic enzymes has shown significant potential in improving glucose release from glucan compared to using either enzyme separately. However, the high-cost production of these enzymes remains a major obstacle to their large-scale application (Novy et al. 2019). Therefore, the effects of enzyme loading were investigated to minimize usage while still maintaining an acceptable glucan conversion. Figure 3 shows the effect of cellulase loading on glucan conversion for three different HBRs, with a fixed dosage of amylolytic enzymes at 5 mg/g substrate. As depicted the figure, increasing cellulase loading from 5 to 40 FPU/g substrate did not result in a significant difference in the 72-h glucan conversion of the IR residue, which remained constant at about 70%. In sharp contrast, the 72-h glucan conversion of the SFR residue increased significantly from 66.87 to 78.41%. Similarly, the 72-h glucan conversion of the GR residue increased from 72.49 to 79.38% as the cellulase loading increased from 5 to 20 FPU/g substrate. Further increase in cellulase loading did not cause significant improvements in glucan conversion. The varying impact of cellulase dosage on glucan conversion among the various HBRs can be partly explained by the difference in their cellulose content. The higher the cellulose content in the substrate, the

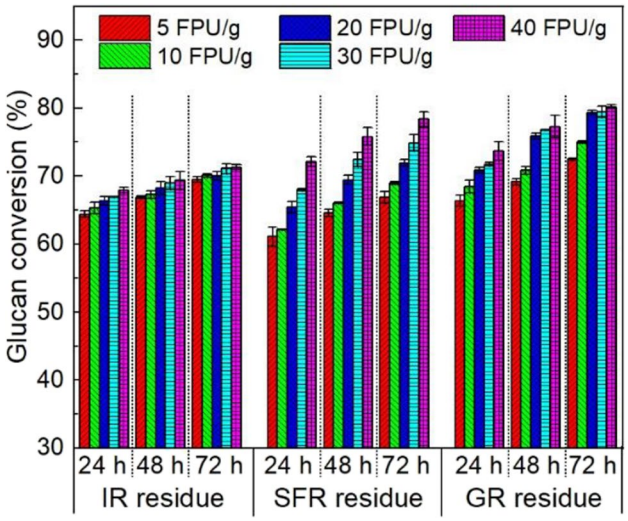

Fig. 3 Effect of cellulase loading on the hydrolysis of the three HBRs using the combined cellulase and amylolytic enzymes. The HBRs were hydrolyzed at 10% (w/v) solids loading using different dosages of cellulase while fixing the loading of amylolytic enzymes at 5 mg/g substrate

higher the required cellulase loading to hydrolyze the cellulose into glucose. For example, the IR residue exhibited the lowest cellulose content of 7.85%, while the SFR residue had the highest cellulose of 21.02% among the tested HBRs (Table 1). As a result, an increase in cellulase loading showed a more pronounced effect on the glucan conversion of the SFR residue compared to the IR residue. To ensure acceptable glucan conversions while considering the high cost of cellulase production, the selected cellulase loading the subsequent hydrolysis experiments was determined as 5, 10, and 5 FPU/g substrate for the IR, SFR, and GR residues, respectively. These loadings maintained a 72-h conversions rate of no less than 70% for all three HBRs.

Figure 4 presents the effect of amylolytic enzymes dosage on glucan conversion for the three HBRs, while keeping the cellulase loading kept at their respective predetermined levels. Similar to the effect of cellulase loading, varying the amylolytic enzymes dosage led to different trend in glucan conversion depending on the used HBRs. As far as the IR residue was concerned, an increase in the loading of amylolytic enzymes from 0.05 to 5 mg/g substrate led to progressive improvement in the 72-h glucan conversion, rising from 54.83 to 70.10%. This observation can be attributed to the high starch content present in the IR residue, which requires a higher dosage of amylolytic enzymes for effective digestion. In contrast, for the SFR and GR residues, a dosage of 0.5 and 1 mg/g substrate of amylolytic enzymes was sufficient to reach high 72-h glucan conversions of 67.91% and 73.44%, respectively. Further increasing the concentration of amylolytic enzymes did not yield a dramatical enhancement

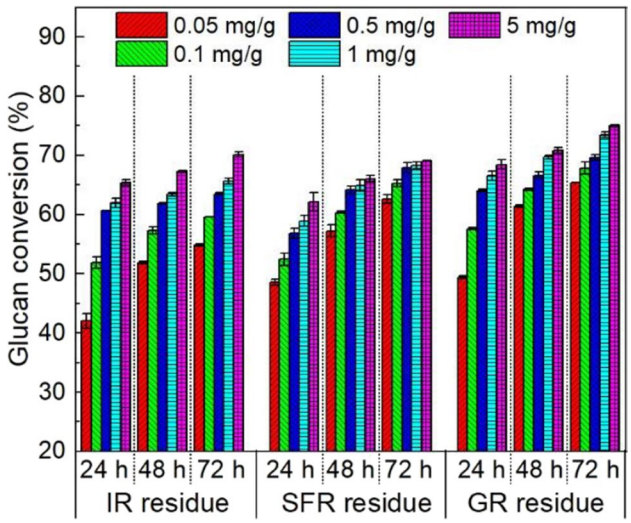

Fig. 4 Effect of amylolytic enzymes dosage on the hydrolysis of the three HBRs using the combined cellulase and amylolytic enzymes. The HBRs were hydrolyzed at 10% (w/v) solids loading with different dosages of amylolytic enzymes while fixing the cellulase loadings at 5, 10, and 5 FPU/g substrate for the IR, SFR, and GR residues, respectively

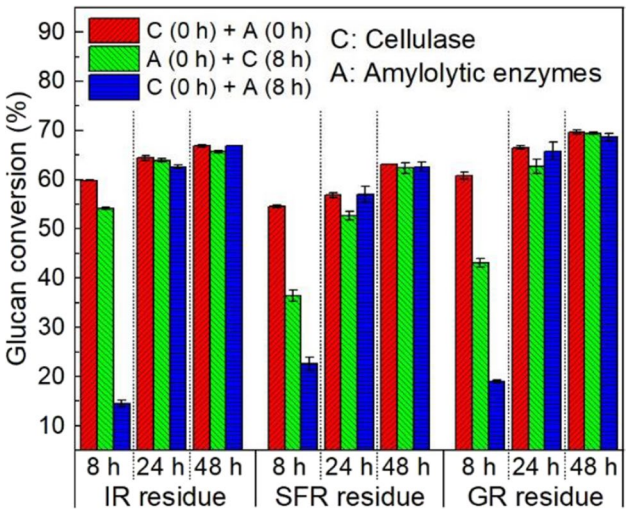

Fig. 5 Effect of sequential addition of cellulase and amylolytic enzymes on the hydrolysis of the three HBRs. The HBRs were hydrolyzed at 10% (w/v) solids loading using combined cellulase and amylolytic enzymes. The cellulase loadings were 5, 10, and 5 FPU/g substrate for the IR, SFR, and GR residues, respectively. Dosages of amylolytic enzymes were 5, 0.5, and 1 mg/g substrate for the IR, SFR, and GR residues, respectively

in glucan conversion for both residues. Based on these findings, the subsequent experiments were conducted using amylolytic enzymes loading of 5, 0.5, and 1 mg/g substrate for the IR, SFR and GR residues, respectively.

In the aforementioned discussions, cellulolytic and amylolytic enzymes were supplemented concurrently to initiate the hydrolysis reaction. Figure 5 shows the influence of sequentially adding both enzymes, where one enzyme was added initially, and another enzyme was introduced after 8 h of hydrolysis, on glucan hydrolysis of the three HBRs. At a short reaction time of 8 h, the concurrent addition of both enzymes at the beginning of the reaction showed a remarkedly superior effect compared to the separate use of either enzyme, once again corroborating their synergistic effect. Simultaneously, hydrolysis of HBRs with amylolytic enzymes alone brought about significant increase in glucan conversion compared to using only cellulase, suggesting that the used cellulase possessed a limited ability to depolymerize starch component in the HBRs. The extent of increase in 8-h glucan conversion depended on the specific HBR used. The IR residue showed the highest increase (3.72 times), which can be attributed to its high starch content that can be easily digested by amylolytic enzymes. Conversely, the SFR residue showed the lowest increase (1.62 times). This observation can be partially explained by the high cellulose content in the SFR residue, as cellulose has been reported to strongly inhibit α-amylase activity (Shokrkar and Ebrahimi 2018).

As the hydrolysis time was extended to 24 h, the order of enzyme addition did not significantly affect glucan conversion of the IR residue compared to their simultaneous addition. However, in the case of the SFR and GR residues, the sequential addition of amylolytic enzymes followed by cellulase resulted in substantially lower glucan conversion compared to the initial simultaneous addition of both enzymes and reverse order of enzyme addition. No significant difference was observed in glucan conversion between the latter two cases. These findings might be explained by the markedly slow reaction rate of enzymatic cellulose hydrolysis, which requires long time to achieve high glucan conversion. This assumption was confirmed when the hydrolysis time was further increased to 48 h, as the change in enzyme addition order did not result in significant difference in glucan conversion regardless of the HBR used for hydrolysis.

The effect of non-ionic surfactants on glucan conversion in the HBRs

In order to reduce the hydrolysis cost of lignocellulosic biomass, researchers have explored the addition of non-ionic surfactants, such as PEG and Tween 20, to the hydrolysis process. These surfactants have been found to greatly improve the rate and yield of enzymatic cellulose hydrolysis. The mechanism behind this phenomenon is due to several factors, including the reduction in the non-productive adsorption of cellulase on hydrophobic lignin, improved stability of cellulase, and increased accessibility of cellulose (Sánchez Muñoz et al. 2022). Although there is limited literature on the effect of

non-surfactants on starch hydrolysis by amylolytic enzymes, previous studies on the influence of non-surfactants on activities of amylases have yielded contradictory conclusions, depending on the specific amylase source (Emampour et al. 2015). Given the beneficial effects of non-ionic surfactants on enzymatic cellulose hydrolysis and the uncertain impact on starch hydrolysis, the effects of two ionic surfactants, PEG6000 and Tween 20, on enzymatic hydrolysis of starch-rich HBRs were investigated using the combined enzymes. The results, depicted in Fig. 6a–c, indicate that neither PEG 6000 nor Tween 20 contributed to improved glucose release from the HBRs within the investigated concentration range. Similarly, investigations of the influences of these two non-ionic surfactants on enzymatic hydrolysis of HBRs using separate cellulase and amylolytic enzymes also came to the same conclusion (data not shown). These findings contradicted the established fact that non-ionic surfactants facilitate enzymatic cellulose hydrolysis. It has been reported that the extent of enhancement in the enzymatic cellulose hydrolysis with addition of surfactants is highly dependent on the lignin content in the substrate (Nogueira et al. 2022). Non-ionic surfactants primarily exert their facilitating effect by competing with cellulase for adsorption on lignin, thus making more free cellulase available for hydrolyzing the substrate. Therefore, the

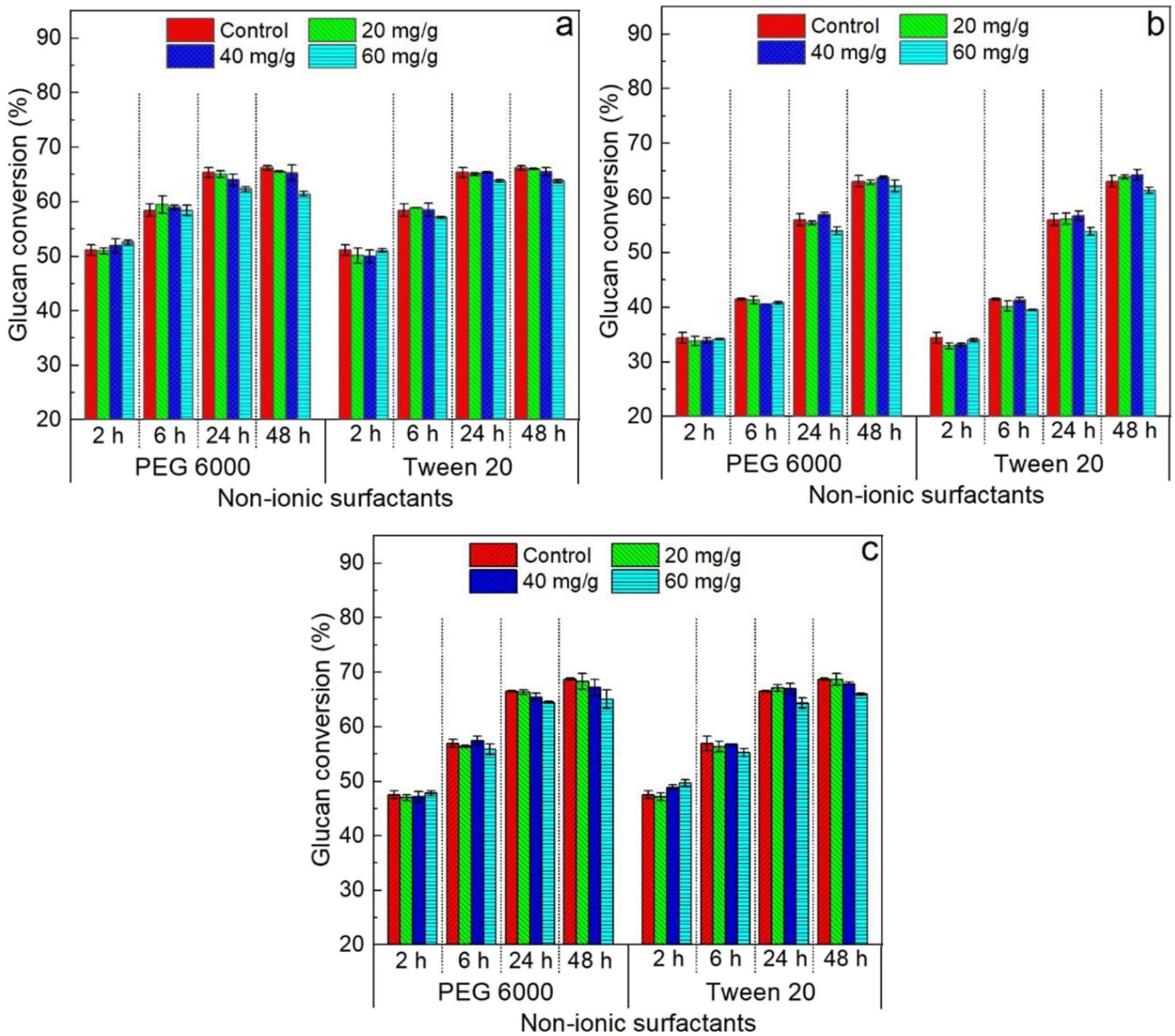

Fig. 6 Effect of non-ionic surfactants on the enzymatic hydrolysis of IR (**a**), SFR (**b**), and GR (**c**) residues using combined cellulase and amylolytic enzymes. The HBRs were hydrolyzed at 10% (w/v) solids loading. The cellulase loadings were 5, 10, and 5 FPU/g substrate for the IR, SFR, and GR residues, respectively. Dosages of amylolytic enzymes were 5, 0.5, and 1 mg/g substrate for the IR, SFR, and GR residues, respectively

higher the lignin content in the substrate, the greater the beneficial effect of non-ionic surfactants on enzymatic cellulose hydrolysis. In this study, the marginal effect of the two tested non-ionic surfactants could be attributed to the relative low lignin content in the three HBRs, ranging from 6.69 to 11.68% (Table 1). On the other hand, since raw non-pretreated HBRs were used for saccharification, it is possible that most of the lignin fraction of the three HBRs was not exposed. As a result, the lignin fraction was unable to non-productively adsorb a significant amount of enzymes. This may explain the absence of improvement in the enzymatic digestion of the HBRs when the two tested non-ionic surfactants were added.

Interestingly, Fig. 6a–c shows that at a high concentration of 60 mg/g substrate, PEG 6000 and Tween 80 seemed to decrease enzymatic glucan hydrolysis of the three HBRs after 24 h. This adverse effect is likely due to the disappearance of surfactant alignment at the air–liquid interface, which is caused by the formation of micelles as their concentration was higher than their respective critical micelle concentration. This increased exposure of the enzymes to the air–liquid interface under agitation, can deactivate cellulase and amylolytic enzymes, resulting in decreased enzymatic glucan hydrolysis. Similar observation was also reported by Mukasekuru et al. (2018).

High-solids fed-batch enzymatic hydrolysis of the HBRs

To achieve a high glucose concentration while maintaining an acceptable glucose yield, high-solids fed-batch enzymatic hydrolysis of HBR was investigated. Four fed-batch modes were tested to achieve a final 30% (w/v) solid loading for the three HBRs under their respective enzyme loadings. According to a review by da Silva et al. (2020), for the fed-batch hydrolysis process, the addition of cellulolytic and amylolytic enzymes in their entirety at the onset of enzymatic hydrolysis yielded better results compared to split addition. Split addition of enzymes, on the other hand, was found to be ineffective or even led to a decrease in the hydrolysis yield. As shown in Table 2, the effectiveness of the different fed-batch modes varied depending on the used HBR. When the IR residue was used for enzymatic digestion, there were no significant differences in glucose production and glucan conversion among the four fed-batch modes, regardless of the hydrolysis time. This can be attributed to the substantial amount of starch present in the IR residue, which is readily digested by amylolytic enzymes, leading to a hydrolysate with a low viscosity that facilitates the glucose release.

When it came to fed-batch hydrolysis of the SFR and GR residues, the feeding modes with early and high-frequency feeding of a low amount of substrate (modes 1 and 3) were observed to enhance enzymatic hydrolysis compared to the modes with late and low-frequency feeding of a high amount of substrate (modes 2 and 4) at a short hydrolysis time of 48 h. This observation could be explained by two factors mentioned in the review by da Silva et al. (2020). Firstly, early feeding favors effective enzymatic hydrolysis by reducing the progressive loss of enzymatic activity that occurs with prolonged hydrolysis time, which was caused by the non-productive adsorption of enzymes on the lignin fraction; Secondly, high-frequency feeding with a low amount of substrate helps maintain a low viscosity

Table 2 High-solids enzymatic hydrolysis of the three HBRs using fed-batch strategy

HBRs	Feeding strategy (%)							Enzymatic hydrolysis for glucose production			
								48 h		96 h	
	Mode	0 h	3 h	4 h	6 h	8 h	9 h	Conc. (g/L)	Conversion (%)	Conc. (g/L)	Conversion (%)
IR	1	10	7		7		6	124.86 ± 1.50	51.85 ± 0.11	128.90 ± 2.24	53.53 ± 0.15
	2	10		10		10		125.59 ± 1.57	52.16 ± 0.36	131.62 ± 1.82	54.75 ± 1.38
	3	15	5		5		5	127.97 ± 2.76	53.19 ± 0.33	130.32 ± 2.90	54.20 ± 0.39
	4	15		7.5		7.5		125.70 ± 0.82	52.21 ± 0.35	130.90 ± 1.08	54.45 ± 0.46
SFR	1	10	7		7		6	92.72 ± 2.50	53.85 ± 1.52	103.80 ± 1.62	60.59 ± 0.98
	2	10		10		10		87.51 ± 2.65	50.69 ± 1.62	97.83 ± 1.95	56.98 ± 1.21
	3	15	5		5		5	92.06 ± 1.61	53.45 ± 0.36	102.62 ± 2.40	59.71 ± 0.24
	4	15		7.5		7.5		85.81 ± 1.19	49.64 ± 0.73	93.71 ± 1.80	54.45 ± 0.49
GR	1	10	7		7		6	76.76 ± 2.21	56.95 ± 1.69	83.15 ± 2.50	61.90 ± 1.92
	2	10		10		10		73.92 ± 1.88	54.77 ± 1.44	82.81 ± 2.95	61.09 ± 2.26
	3	15	5		5		5	76.43 ± 0.46	56.64 ± 0.35	81.93 ± 2.02	60.68 ± 1.54
	4	15		7.5		7.5		73.35 ± 2.13	54.34 ± 1.63	82.81 ± 2.09	61.57 ± 1.60

The HBRs were hydrolyzed with combined cellulase and amylolytic enzymes. Cellulase loadings were 5, 10, and 5 FPU/g substrate for the IR, SFR and GR residues, respectively. Dosages of amylolytic enzymes were 5, 0.5, and 1 mg/g substrate for the IR, SFR, and GR residues, respectively

in the hydrolysate, facilitating enzymatic glucan hydrolysis. However, with an extended hydrolysis time of 96 h, the effect of fed-batch modes on enzymatic hydrolysis of the SFR residue remained the same, where modes 1 and 3 yielded better hydrolysis results than modes 2 and 4. Moreover, there were no significant differences in glucose concentration and glucan conversion between the two modes within the same category at hydrolysis times of 48 and 96 h (mode 1 vs. mode 3, and mode 2 vs. mode 4). In the case of the GR residue, the four fed-batch modes did not exhibit a significant difference in enzymatic hydrolysis even after 96 h of reaction. This result can be attributed to the higher starch content and lower cellulose content in the GR residue compared to SFR residue (Table 1). As mentioned earlier, starch can be hydrolyzed at a faster rate than cellulose. Therefore, when considering a substrate concentration as high as 30% (w/v), it is highly probable that a prolonged hydrolysis time of 96 h enables the hydrolysis of both digestible starch and cellulose in all four fed-batch modes. This resulted in nearly identical glucose production levels across the different modes.

After only 48 h of fed-batch hydrolysis, the IR and SFR residues reached glucose concentrations of around 125 g/L and 92 g/L, respectively. Furthermore, after 96 h of fed-batch hydrolysis, the GR residue achieved a glucose concentration of about 83 g/L. According to reports by Cheng et al. (2020) and Hernández-Beltrán et al. (2021), the economically feasible large-scale production of ethanol from lignocellulosic biomass requires a minimum fermentable sugar concentration of 80 g/L in the hydrolysate. Remarkably, the fed-batch enzymatic hydrolysis of these raw non-pretreated HBRs yielded glucose concentrations exceeding 80 g/L, indicating their suitability as ideal substrates for biofuel production. In addition, even when utilizing a high substrate loading of 30% (w/v), the three raw non-pretreated HBRs demonstrated remarkable glucan conversion rates. The IR residue achieved an impressive glucan conversion of around 54%, while both SFR and GR residues exhibited even higher rates of about 61% after 96 h of hydrolysis. These glucan conversion rates were found to be comparable or even superior to the results obtained from differently pretreated conventional lignocellulosic biomass under identical substrate loading conditions (Chen and Liu 2017; Modenbach and Nokes 2013).

Conclusion

Three starch-rich raw HBRs underwent batch and fed-batch enzymatic hydrolysis to produce glucose. The combined use of cellulase and amylolytic enzymes effectively depolymerized glucan, yielding higher glucose compared to individual enzymes. High glucan conversion ($\geq$ 70%) was achieved with low dosages of cellulase and amylolytic enzymes at 10% (w/v) solids loading. Supplementation with PEG 6000 and Tween 20 had no saccharification improvement. Fed-batch hydrolysis at 30% (w/v) solids produced impressive glucose concentrations of 125, 92, and 83 g/L for IR, SFR, and GR residues, respectively. These results highlight the potential of starch-rich HBRs in commercial biorefinery processes.

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Author contribution Benkun Qi conceived and designed the study and wrote the first draft of the manuscript. Zhenzhou Zhu and Sirong Wu performed the experiments. Zhenzhou Zhu and Caixia Wang conducted the data analysis. Jianquan Luo and Yinhua Wan critically reviewed the manuscript. All the authors read and approved the final manuscript.

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Data availability The datasets and materials used in the current study are available from the corresponding author on reasonable request.

Declarations

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Consent for publication Not applicable.

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