



Fed-batch enzymatic hydrolysis of plantain pseudostem to fermentable sugars production and the impact of particle size at high solids loadings

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Abstract

In this work, the performance of a fed-batch high solids enzymatic hydrolysis of plantain pseudostem was studied at different particle sizes. The plantain pseudostem was pretreated with an alkaline hydrogen peroxide medium. Two set of experiments were carried out in a stirred tank bioreactor at substrate concentrations of 10% and 15% w/v, combined each one with particle sizes #20–40, #40–60, and # > 100, in 1 L of reaction volume. Particle sizes of #20–40 and #40–60 allowed around 99% of holocellulose conversion with a substrate concentration of 10% w/v, and 91% with 15% w/v; meanwhile enzymatic hydrolysis at particle size of mesh # > 100 yielded 70% and 73%, with a substrate concentration of 10% and 15% w/v, respectively. The conditions recommended for enzymatic hydrolysis at high solids of 15% w/v through fed-batch operation are particle size #20–40 and 6 h of reaction, achieving 91% of holocellulose conversion and 82 g/L of reducing sugars.

Keywords Plantain pseudostem · Mechanical pretreatment · Alkaline hydrogen peroxide · Enzymatic hydrolysis · Fed batch · High solids

1 Introduction

Today, the use of renewable sources for production of energy and society demands such as biofuels and value-added

Highlights

- AHP pretreatment did not decrease the lignin content on plantain pseudostem.
- Particle sizes #20–40 and #40–60 yielded 99% of holocellulose conversion.
- Particle size #>100 yielded 73% of holocellulose conversion.
- Fed-batch operation allowed homogeneous mixing at high-solids of 15% w/v.
- Particle size #20–40, 6 h of reaction and 15% w/v of solids were the best conditions.

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products has gained an increasing interest [1]. This issue brings into play the environmental concerns in the reduction of CO₂ emissions, being the biofuels one of the key for alternative energy in CO₂ mitigations [2]. The lignocellulosic biomass is a promising renewable source that can be converted into fermentable sugars for production of biofuels or high-value added chemicals [3, 4]. In the production chain of fermentable sugars in a second-generation scenario, two main steps appear: (i) pretreatment to disrupt the recalcitrant lignocellulose structure and decreasing the cellulose crystallinity make accessible the enzymes [5, 6], and (ii) enzymatic hydrolysis to obtain fermentable reducing sugars through the action of cellulases and hemicellulases enzymes on pretreated lignocellulosic biomass [7]. After this, fermentation processes for reducing sugars conversion is crucial because it allows for production of various groups of substances under relatively mild conditions [8].

Banana is the common name for herbaceous plants of the genus *Musa* L. family *Musaceae* [9] and is the second most produced fruit in the world. The worldwide annual production is approximately of 125 million tons, which generates about 250 million tons of fresh lignocellulosic biomass residues [10]. Approximately 30 million tons of banana production corresponds to plantains [11], i.e., several banana and plantain

varieties are produced in Colombia, sharing the first positions of agricultural economy with coffee, palm oil, sugar cane, and flowers [12].

The plantain pseudostem serves very little industrial purposes, and it is left to rot away in their plantations due to its relatively very low appeal to grazing livestock as a result of high oxalate content. Consequently, the practice to utilize the plantain pseudostem for production of fermentable sugars ensures another source for production of renewable energy or high-value added chemicals [13]. Moreover, there is a lack of studies according the potential of plantain wastes and the majority of the studies have been focused in the peel and fruit waste [14]. Plantain pseudostem is more fibrous in nature, and this aspect limits the rate of enzymatic hydrolysis process due to its high degree of polymerization and crystallinity [15]. For this reason, the pretreatment must guarantee a high recovery of cellulose and hemicellulose and increase their digestibility prior the enzymatic hydrolysis process [16], being alkaline hydrogen peroxide (AHP) a highly effective during the pretreatment of several feedstocks [17], but there is a scarcity of studies in application of AHP pretreatment on banana non-food wastes.

Particle size plays a vital role due its influences affecting the reaction rate, mass transfer, effectiveness of pretreatment, and enzymatic hydrolysis on sugars yield and consequent fermentation processes [18]. However, the literature contains inconsistent reports on correlation the reduction of biomass particle size with enzymatic hydrolysis sugars yield [19]. Otherwise, optimization of lignocellulosic particle size is essential for achieving high sugars conversion and low production cost [20].

In enzymatic hydrolysis processes, the biomass loading in a reactor with a concentration greater than 15% w/v is considered as a high solids process, in which a little amount of free water is available for mass transfer [21]. Besides the high solids processing represents a strategy to obtain a high concentration of reducing sugars [22]. For example, for bioethanol production using yeast like *Saccharomyces cerevisiae*, it is necessary to reach at sugar concentration of 80 g/L and thus the process be able to obtain the minimum ethanol concentration of 40 g/L to start an economic viability in purification process [23, 24]. In addition, enzymatic hydrolysis at high solids is a feasible process because it provides high productivity as result of lower energy consumption and water input, also operating cost of hydrolysis and subsequent fermentation [25].

On the other hand, the batch operation is the typical and most used way to reach high solids loadings; however, it suffers of a poor mixing, consequently a difficult adjustment of pH and temperature, and it may lead lower performance of the reaction [26, 27]. Even more, the enzymatic hydrolysis carried out at biomass concentration of 10% w/v is somewhat difficult in the sense of mixing and therefore adjustment of pH and temperature. In fact, mixing problems can be observed on

enzymatic hydrolysis experiments of lignocellulosic biomass concentration at > 7% w/v in batch operation. Therefore, the fed-batch strategy is an option that guarantees a good mixing in the process [24].

One of the strategies to perform the fed-batch operation is starting the enzymatic hydrolysis experiments with a moderate solid loading and frequently adding more solids after certain reaction times, i.e., de Godoy et al. [21] started their experiments at 10% w/v of solids concentration and then added 5% w/v of solids and enzyme load every 6 h of hydrolysis until reach the final concentration of 25% w/v with a glucan conversion of 67%. Meanwhile, Hernández-Beltrán and Hernández-Escoto [28] performed another strategy that consisted in adding the whole enzyme amount in the beginning of enzymatic hydrolysis and then added small amounts of solids (2.5 g on dry basis) every 5 min until reach the final concentration of 20% w/v with a sugars conversion of 75% after 7 h of reaction time.

This study consisted into provide information about the influence of AHP pretreatment on plantain pseudostem using three particle sizes. The fed-batch strategy was used to perform the enzymatic hydrolysis experiments at 10% and 15% w/v of solids loadings. Subsequently, the approach lies into determine the best process conditions for fermentable sugars production according the particle size of mesh, reaction time, biomass concentration, and enzyme complex load.

2 Materials and methods

2.1 Raw material and pretreatment

The plantain pseudostem (PS) was collected from Chinchiná, Caldas, Colombia, and it was received in big pieces. The PS was cut into small pieces around 1 in. with a stainless-steel knife and was put to dry at 60 °C. Then, PS was milled by using Thomas-Wiley® Laboratory Mill and sieved between mesh #20–40, #40–60, and # > 100.

The AHP pretreatment was carried out in duplicate at a reaction volume of 1 L (the total volume of the reactor used was 2 L). The reaction volume of 1 L consisted in a mixture of 6% w/v of PS and distilled water, mechanically stirred at 100 rpm; then, 2% v/v of sodium hydroxide (NaOH), and 2% v/v of hydrogen peroxide (H₂O₂) were added. The reaction was carried out at 60 °C, maintaining a pH of 11.5 during 5 h. Then, the pretreated plantain pseudostem (PSA) was filtered in a commercial cloth mesh and washed with 1 l of deionized water, and an adjustment of pH approximately 5.0 with dilute sulfuric acid was made [29].

2.2 Enzymatic microhydrolysis

To determine the effective process conditions of the substrate-enzyme system, for scale-up of the enzymatic hydrolysis

process in bioreactor system, a microreaction technique was followed. Hernández-Beltrán et al. [30] proposed microhydrolysis experiments varying process conditions such as temperature, pH, and amount of enzyme complex due the manufacturer of enzyme complex recommend carrying out a response curve to determine the optimal process conditions.

The microreaction consisted into use microtubes as microreactors: each one was filled with a volume of 1.5 mL of a suspension of 1% w/v of substrate. The experimentation was carried out in a shaking thermoblock of 24 places (Thermomixer-comfort by Eppendorf®) with an agitation of 900 rpm.

The enzyme complex used was Cellic® CTec2 (CT2), kindly provided by Novozymes® Latin America Ltda. (Rua Professor Francisco Ribeiro, Araucária - Paraná, Brazil).

Full factorial design of experiments in triplicate with three levels for temperature (T) and pH and four levels for enzyme loading (EL) were as follows: $T = \{45, 50, 55\}$ °C, $pH = \{4.5, 5.0, 5.5\}$, $EL = \{1.0, 3.0, 5.0, 7.0\}$ $\mu\text{L}/0.015$ g-PSA. The response surface methodology (RSM) was used to determine the optimal combination of process conditions in the substrate-enzyme system PSA-CT2.

2.3 Enzymatic hydrolysis of 1 L through fed-batch operation

The reaction system used was an Applikon® BioBundle stirred tank bioreactor with automatic control of temperature and pH; the stirring was mechanically performed at 150 rpm with a turbine propeller.

The enzymatic hydrolysis experiments were carried out in fed-batch operation, at reaction volume of 1 L and processing time of 24 h, using three different particle sizes (mesh #20–40, #40–60, and # > 100) and two biomass concentrations (10% and 15% w/v); therefore, six systems of reaction were considered.

The fed-batch operation consisted in the preparation of an initial bulk of the corresponding distilled water plus the whole enzyme load. Then, the addition of 5 g of PSA every 10 min until reach the final PSA concentration was addressed. Samples were taken out frequently to generate the kinetic trajectories.

2.4 Analytical methods

The chemical composition for cellulose, hemicellulose, and lignin of the PS and PSA was determined following the established technique by National Renewable Energy Laboratory (NREL): Preparation of samples for compositional analysis [31], determination of total solids in biomass, and total dissolved solids in liquid process samples [32], determination of ash in biomass [33], determination of extractives in

biomass [34], and determination of structural carbohydrates and lignin in biomass [35].

To measurement the enzymatic activity of cellulases, the NREL technique was followed [36].

The reducing sugars measured were glucose, xylose, mannose, galactose, and arabinose. The measurement of reducing sugars and as well the furfurals and hydroxymethylfurfurals (HMF) were carried out in HPLC Elite Lachrom equipped with CHO-782 Pb column, refractive index (RI) as detector type, a flux of 0.6 mL/min, detector temperature of 45 °C, oven column temperature of 80 °C, and water type I as mobile phase or eluent.

3 Results and discussion

3.1 Chemical composition of PS and PSA

The chemical characterization of PS and PSA is given in Table 1. PS had 34.7% (w/w) of cellulose and 10.1% (w/w) of hemicellulose, meaning 44.8% (w/w) of holocellulose composition, versus a 30.8% (w/w) of lignin one. In the literature, the amount of lignin varies, according to whether it is the plantain or banana pseudo-stem or peel, i.e., Amadi and Ifeanacho [13] reported cellulose content in plantain pseudo-stem, of 25.5% (w/w); Idrees et al. [37], Gabhane et al. [15], and Kusmiyati and Sukmaningtyas [38] reported cellulose content in banana pseudostem of 46%, 44.3%, and 28.8% (w/w), respectively; Igbokwe et al. [39] and Nwabanne and Aghadi [40] reported cellulose content in plantain and banana peels of 46% and 65% (w/w), respectively.

One goal among others of pretreatment process is the recalcitrance decreasing of lignocellulosic material by removing lignin [41, 42]; however, according to PSA, the lignin composition resulted in 27.6% (w/w), just lignin composition decreased by 3% (w/w) after AHP pretreatment. On the other hand, extractives and ash contents in PS decreased in 9% and 6% (w/w) respectively according to PSA, affecting positively the cellulose increase by 17% (w/w), meanwhile the hemicellulose was kept in similar quantity, 10.1% (w/w) for PS and 11.2% (w/w) for PSA. Shimizu et al. [43] found that lower alkaline conditions, i.e., 5% and 15% of alkali reagent concentration, resulted in little changes of chemical composition, and they suggested to carry out severe pretreatment with higher concentration of alkali and oxidant reagents, i.e., alkali reagent concentration greater than 25% and hydrogen peroxide concentration of 8%; Hideno [44] as well, performed the AHP pretreatment on rice straw with NaOH concentration of 8% and H₂O₂ concentration of 16% v/v finding a cellulose content > 70% (w/w). However, the cost-benefit compromise by using high concentrations of alkali and oxidant reagents must be addressed under an engineering point of view. Alvarez-Vasco and Zhang [45] found that alkaline hydrogen

Table 1 Chemical composition of untreated and pretreated plantain pseudostem

Lignocellulosic material	Component on dry basis % w/w				
	Cellulose	Hemicellulose	Lignin	Extractives	Ash
PS	34.7 ± 0.5	10.1 ± 0.1	30.8 ± 0.3	14.6 ± 1.2	9.7 ± 0.1
PSA	52.3 ± 0.2	11.2 ± 0.1	27.6 ± 0.5	5.1 ± 2.5	3.7 ± 0.1

peroxide pretreatment at low peroxide loadings achieved up to 95% cellulose-to-glucose yield for softwood.

Although lignin content in PSA was significant and can cause inhibition in the catalytic action of cellulolytic enzymes [46], it must be recalled that the important aspect is the easiness of holocellulose conversion to fermentable sugars [47]. Salehian et al. [48] reported a nonlignin-removal in some scenarios such as the one in which they used a pretreatment method with NaOH at 100 °C and 60 min of retention time. It was observed that the use of alkali reagent in pretreatment method significantly reduced the crystallinity of cellulose that can lead a positive performance in further steps. As well, Lai et al. [49] noted a non-reduction of lignin content at many scenarios that they tested the pretreatment with NaOH and reported an enhancement in enzymatic hydrolysis due the modification of lignin.

3.2 Effective process conditions for enzymatic hydrolysis in stirred tank reactor

The response surface model obtained from microhydrolysis experiments of the substrate-enzyme system PSA-CT2, where Y is the yield of reducing sugars according the total PSA loaded, is the following:

$$\begin{aligned}
 Y = & -706 + 7.52 \times EL + 56.6 \times pH + 24.86 \\
 & \times T - 0.404 \times EL^2 - 4.01 \times pH^2 - 0.2680 \\
 & \times T^2 - 0.548 \times EL \times pH + 0.0327 \times EL \\
 & \times T - 0.054 \times pH \times T \quad (1)
 \end{aligned}$$

The standard deviation of the experiments in the full factorial design was 4.4%, and model adjusted r squared of 89%. The optimal process conditions predicted by the quadratic model (1), in which the system PSA-CT2 reaches its maximum reducing sugars yield of 69% in 1 h of processing time, are $EL = 7.0 \mu\text{L/g-PSA}$, $pH = 5.5$, and $T = 46.31 \text{ }^\circ\text{C}$. The enzyme complex amount is high considering the subsequent scaling-up of enzymatic hydrolysis experiments at reaction volume of 1 L; therefore, another effective combination of process conditions was found by the response surface model (1) in which the yield not decreasing significantly but the enzyme complex amount does.

The response surface model (2) was evaluated at optimal pH and temperature but analyzing the range of values from

1.0–7.0 $\mu\text{L}/0.015 \text{ g-PSA}$ resulting a yield of 61% and the amount of enzyme complex is significantly lower than the optimal one.

CT2 possessed 200 FPU/mL of enzyme activity. It means that the enzyme complex amount determined of 3.0 $\mu\text{L}/0.015 \text{ g-PSA}$ corresponds to 40 FPU/g.

3.3 Fed-batch enzymatic hydrolysis in bioreactor of 1 L

In advance, the measurement for inhibitory compounds like furfurals and HMF was performed, showing zero concentrations in both compounds. Furthermore, if a subsequent fermentation process were taken out, the inhibitory compounds will not affect it.

3.3.1 Fed-batch enzymatic hydrolysis at 10% w/v of solids

Table 2 shows the outcomes of enzymatic hydrolysis experiments carried out with PSA at 10% w/v, with the three different particle sizes in bioreactor at reaction volume of 1 L. Enzymatic hydrolysis with particle sizes of mesh #20–40 and #40–60 achieved the same reducing sugars yield of 99% according the holocellulose content after 24 h of reaction time. Both particle sizes reached a glucose concentration approximately 52 g/L and xylose concentration around 10 g/L. Our study demonstrated that glucose concentration was higher than 30 g/L which was obtained by Kusmiyati and Sukmaningtyas [38] using banana pseudostem in the enzymatic hydrolysis process at solids concentration of 10% w/v and 72 h of reaction time.

In Table 2, it can be pointed out that AHP pretreatment was more aggressive on hemicellulose with the smaller particle size (mesh # > 100) since the glucose and xylose concentration decreased considerably, achieved a significantly lower glucose and xylose concentrations, of 40 g/L and 2.7 g/L respectively.

Figure 1 depicts the evolution of experiments in terms of yield of reducing sugar with respect to holocellulose. It can be noted that the addition of 5 g of PSA (on dry basis) every 10 min allowed the enzyme complex to liquefy the substrate; in this way, the reaction was able to contain more solids due the increase in the availability of water in the bulk. The PSA dosing reached the concentration of 10% w/v in 3.3 h.

In Fig. 1, it can be observed that experiment with particle size of mesh #20–40 yielded 92% of its consequent total performance

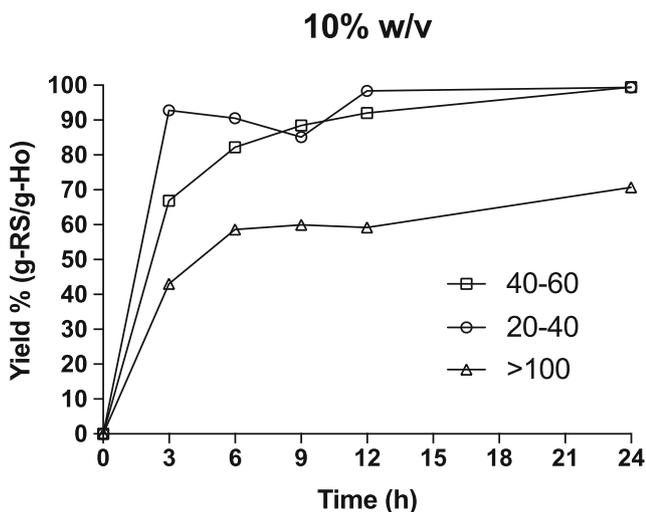
Table 2 Reducing sugars concentrations and their yields in fed-batch experiments at 10% w/v of pretreated plantain pseudostem. *G* glucose, *X* xylose, *RS* reducing sugar, *C* cellulose, *He* hemicellulose, *Ho* holocellulose

Particle size of mesh (#)	Glucose (g/L)	Xylose (g/L)	Yield % (g-G/g-C)	Yield % (g-X/g-He)	Yield % (g-RS/g-Ho)	Yield % (g-RS/g-PSA)
20–40	51.02	9.4	99.10	84.98	99.35	63.17
40–60	52.96	10.14	99.26	88.39	99.46	63.23
> 100	40.41	2.7	78.04	24.27	70.68	44.94

of 99% in the first 3 h of reaction time. Meanwhile, the enzymatic hydrolysis of particle size of mesh #40–60 which at the same 3 h of reaction time yielded 66% of its subsequent total performance of 99%. It means the performance was 26% lower, and it needs 9 h more of reaction time to achieve the equal yield of 92%.

On the other hand, the performance of the experiment with particle size of mesh # > 100 is lower than those obtained with the other particle sizes. The reducing sugars yield was 70% according to holocellulose content, meanwhile the glucose and xylose concentrations were 40 and 2.7 g/L respectively. Therefore, the optimal particle size resulted in mesh #20–40.

Enzymatic hydrolysis with particle size of mesh #20–40 achieved its maximum performance at 12 h of reaction time, meanwhile the experiments with particle sizes of mesh #40–60 and # > 100 did it at 24 h of reaction time. According to process engineering point of view, not only the maximum performance of the reaction must be taken into account but also the reaction time it would take to reach the maximum performance. This in view of time savings and quantity of obtained product, which is oriented to optimize the costs of the process [20, 21, 50]. In this way, the bigger particle sizes require less energy such as the particle size of mesh #20–40 compared with the other particle sizes studied in this paper. Besides, the experiment with particle size of mesh #20–40 reached a yield of 92% in 3 h, being similar to the yields of 98% and 99% reached within 12 and 24 h respectively.

**Fig. 1** Enzymatic hydrolysis trajectories of fed-batch experiments at 10% w/v of PSA. RS: reducing sugar; Ho: holocellulose

3.3.2 Fed-batch enzymatic hydrolysis at 15% w/v of solids

Table 3 shows the outcomes of enzymatic hydrolysis experiments carried out at PSA concentration of 15% w/v in terms of the concentrations of glucose and xylose obtained, as well as their yield according the cellulose and hemicellulose contents respectively, and the reducing sugars yield according to the holocellulose and total PSA loaded. It can be observed that for PSA with particle size of mesh #20–40 and #40–60, the reducing sugars yield achieved 91% according to holocellulose content in a reaction time of 24 h, meanwhile with the mesh # > 100, it was 73%. Enzymatic hydrolysis with particle size of mesh #20–40 and #40–60 reached a glucose concentration approximately 70 g/L and xylose concentration around 13 g/L. However, enzymatic hydrolysis with particle size of mesh # > 100 reached 50 g/L of glucose. In addition, it can be noted that AHP pretreatment solubilizes hemicellulose content in particle size of mesh # > 100 four times more compared with other particle sizes due to xylose that presents a decrease from 13 to 3 g/L.

Figure 2 shows the experiments presented in Table 3. In these experiments, the dosing period was of 5 h to reach the final PSA concentration of 15% w/v. It can be observed that in 6 h of reaction time, the experiment with particle size of mesh #20–40 reached a yield of 88%, which had no significant difference with the performance showed at 24 h of reaction time. Meanwhile, the experiment with particle size of mesh #40–60 reached a yield around of 70% in 6 h of reaction time, which means 21% less compared with the one of 24 h of reaction time. In this sense, similar performances of the three particle sizes than enzymatic hydrolysis at 10% w/v of solids are evidenced; also Kapoor et al. [20] found the same effects when working with different particle sizes and with solids concentration of 10% and 15% w/v, concluding that very small particle size does not lead more cellulose conversion, but when the solids concentration increases from 10 to 15% w/v, the yield decreases. In contrast to Shimizu et al. [43], the need for high alkali reagent concentration was avoided due the combination of dilute concentrations of 2% v/v of sodium hydroxide and hydrogen peroxide, and a conversion of holocellulose-to-reducing sugar over 90% in 6 h of reaction time was achieved.

On the other hand, the enzyme complex amount used of 40 FPU/g of PSA is higher than the that of 20 FPU/g as suggested by Guerrero et al. [14]; however, they reported the maximum

Table 3 Reducing sugars concentrations and their yields in fed-batch experiments at 15% w/v of pretreated plantain pseudostem. *G* glucose, *X* xylose, *RS* reducing sugar, *C* cellulose, *He* hemicellulose, *Ho* holocellulose

Particle size of mesh (#)	Glucose (g/L)	Xylose (g/L)	Yield % (g-G/g-C)	Yield % (g-X/g-He)	Yield % (g-RS/g-Ho)	Yield % (g-RS/g-PSA)
20–40	69.97	12.1	91.46	74.01	91.46	58.15
40–60	68.86	13.1	90.22	79.88	91.57	58.22
> 100	49.55	3.2	67.06	20.16	73.41	38.47

yield of enzymatic hydrolysis between 48 and 72 h of processing time, and in 6 h, the glucose concentration is less than the half of the maximum that enzymatic hydrolysis will theoretically reach.

The recommended process conditions for enzymatic hydrolysis were the use of particle size of mesh #20–40, at reaction time of 6 h, enzyme complex amount of 40 FPU/g, and solid concentration 15% w/v since it was validated compared with solids concentrations of 10% w/v due to in both cases, the yield was over 90%; this behavior is explained by the solid effect; since high solids loadings increased, it influences negatively the enzyme adsorption, reducing enzymatic access to the cellulose, and the enzymatic hydrolysis yield decreased [14]. Moreover, these process conditions would lead to perform multiple experiments instead of just carrying out one until reaching the maximum yield. However, it is recommended to carefully optimize the process conditions for each biomass as it may change with the nature of biomass [20, 51].

4 Conclusions

The content of holocellulose in plantain pseudostem is abundant, so it is an agro-residue with high potential to fermentable sugars production. Diluted alkaline oxidative medium makes the holocellulose available for an effective conversion to

reducing sugars. Particle sizes of mesh #20–40 and #40–60 had positive effect in terms of holocellulose conversion compared with particle size of mesh # > 100. Fed-batch operation allowed homogenous mixing throughout enzymatic hydrolysis experiments at solids concentration of 10% and 15% w/v together with three different particle sizes analyzed. The recommended combination of the factors resulted in solid concentration of 15% w/v, particle size of mesh #20–40, 6 h of reaction time, and enzyme complex amount of 40 FPU/g, allowing a holocellulose conversion of 91% and reducing sugar concentration of 82 g/L.

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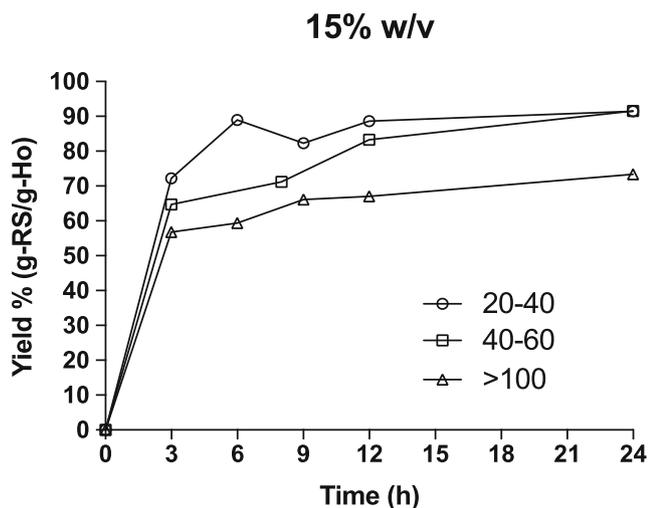


Fig. 2 Enzymatic hydrolysis trajectories of fed-batch experiments at 15% w/v of PSA. RS: reducing sugar; Ho: holocellulose

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