BioEnergy Research (2019) 12:81–93
https://doi.org/10.1007/s12155-018-9956-6

Addition of Soybean Protein Improves Saccharification and Ethanol Production from Hydrothermally Pretreated Sugarcane Bagasse

Camila Florencio[1] · Alberto C. Badino[2] · Cristiane S. Farinas[1,2]

Published online: 3 January 2019

Abstract

The bioconversion yield of ethanol from lignocellulosic feedstocks is negatively affected by the unproductive adsorption of cellulolytic enzymes onto lignin. In this work, soybean protein was used as a lignin-blocking additive, with the aim of improving the production of ethanol from enzymatic hydrolysates of pretreated sugarcane bagasse. Investigation was made of the effects of the type of hydrothermal pretreatment process—steam explosion (SE) or liquid hot water (LHW), loadings of solids and enzymes, and bioreactor type. The addition of soybean protein led to a exceptional 76% increase of glucose released using the LHW pretreated bagasse, after 24 h of reaction, employing a high-solids loading (15%, *w*/*v*) and a low enzyme dosage (5 FPU/g dry biomass). A significant improvement was also achieved for industrial-like mixing conditions in a bench-scale stirred tank reactor, increasing the glucose released by 61 and 42% for the LHW and SE processes, respectively. Ethanol production was also positively affected by the presence of soybean protein, with increases of up to 86 and 65% for the LHW and SE hydrolysates, compared to the control experiment. Characterization of the sugarcane bagasse after the adsorption of soybean protein, using Fourier transform infrared spectroscopy (FTIR) and scanning electron microscopy (SEM), corroborated the higher affinity of the additive for the LHW bagasse. These findings suggest that soybean protein supplementation during enzymatic hydrolysis by commercially available enzymes is an effective strategy for achieving higher saccharification yields from hydrothermally pretreated biomass, hence improving ethanol production.

Keywords Enzymatic hydrolysis · Lignin · Unproductive adsorption · Lignocellulosic biomass · Sugarcane bagasse · Soybean protein

Introduction

Lignocellulosic ethanol is an important alternative renewable energy resource that can contribute to the progressive reduction of fossil fuel use, which is required for the implementation of a sustainable bioeconomy. Furthermore, ethanol from lignocellulosic feedstocks can make important contributions to rural economic development and enhanced sustainability of agricultural landscapes in both developed and developing economies [1]. In these ethanol production processes, pretreatment of the biomass is required in order to overcome the natural recalcitrance of the lignocellulosic material, increasing its biodegradability by deconstructing the plant cell wall structure [2, 3].

✉ Cristiane S. Farinas
cristiane.farinas@embrapa.br

[1] Embrapa Instrumentação, Rua XV de Novembro 1452, São Carlos, SP 13560-970, Brazil

[2] Graduate Program of Chemical Engineering, Federal University of São Carlos, São Carlos, SP 13565-905, Brazil

Among the different types of pretreatments, hydrothermal techniques such as steam explosion [4] and liquid hot water processes [5] have been applied to a wide range of lignocellulosic feedstocks. In these hydrothermal pretreatments, the water is used at elevated temperature and pressure to modify the lignocellulosic biomass structure in order to increase enzyme accessibility to cellulose and hemicellulose. Fractionation occurs mainly by cellulose hydration and depolymerization of hemicellulose through the penetration of water in the plant cell wall structure [6]. These reactions take place because at high temperature (150–230 °C), the increased ionic strength of water facilitates acid-base catalyzed reactions [7].

Hydrothermal pretreatments have been considered as an advantageous, cost-effective, and environmentally friendly pretreatment processes, since only water is usually used, which avoids addition of catalysts or chemicals and minimizes equipment corrosion problems [6, 8, 9]. However, irrespective of the type of process used, a drawback of the pretreatment step is related to the formation of inhibitors of the subsequent biochemical reactions of hydrolysis and fermentation [10]. Considering the catalytic inhibitors derived from the lignin

fraction, the cellulolytic enzymes can be deactivated/inhibited by the soluble phenolic compounds derived from lignin degradation [11–15], as well as by the residual solid lignin fraction that remains after the pretreatment process [5, 16–18].

The unproductive adsorption of cellulase enzymes onto residual lignin in pretreated lignocellulosic biomass decreases the enzymatic hydrolysis yield by reducing the availability of free cellulases [3, 19]. The type of binding that prevails in this unproductive adsorption process includes hydrophobic, electrostatic, and hydrogen-bonding interactions, which can vary according to the enzyme, lignin properties, and the type of pretreatment [17]. In order to overcome this problem, the addition of lignin-blocking agents during the enzymatic hydrolysis constitutes a potential strategy to improve the efficiency of saccharification and reduce the amount of enzyme lost in the process [5, 16, 17, 20–23]. Additives such as bovine serum albumin (BSA), Tween 20 and 80 surfactants, and polyethylene glycol (PEG) have been studied for their beneficial effects during enzymatic hydrolysis [17, 24, 25].

Alternatively, less expensive additives have been suggested for lignin blocking, including soybean protein. A recent study showed that the addition of soybean protein had a positive effect, with an almost twofold increase in the glucose released during saccharification of pretreated sugarcane bagasse using enzymes produced on-site by filamentous fungi [23]. Enzymatic hydrolysis experiments using different substrates indicated that such improvement observed with the addition soybean protein was mainly due reduction of unproductive adsorption of enzymes onto lignin. Another recent study [26] evaluated the influence of different alternative low-cost additives in the saccharification of sugarcane bagasse using a commercial enzymatic cocktail and found that the addition of soybean protein could also enable a decrease in the reactor operation time by up to 66%. In order to achieve the same hydrolysis yield without the soybean additive, the enzyme loading would need to be increased by 50% [26]. However, investigation is still needed of the way that the use of soybean proteins might affect the hydrolysis of pretreated biomass and the impact of this additive on ethanol production. The elucidation of these issues is of great importance in order to be able to demonstrate the feasibility of such a strategy for use under industrially relevant conditions.

In the present study, soybean protein was used as a lignin-blocking additive, with the aim of improving the efficiency of both enzymatic hydrolysis and ethanol production from pretreated sugarcane bagasse, using a commercial enzymatic cocktail. Investigation was made of the effects of the type of hydrothermal pretreatment process (steam explosion or liquid hot water), loadings of solids and enzymes, and the type of bioreactor. Additional studies were conducted to elucidate the nature of the enzyme-substrate interaction and to characterize the biomass, using FTIR and SEM techniques.

Materials and Methods

Additive

Soybean protein (soybean protein isolate with 90% protein content, from Bremil, Rio Grande do Sul, Brazil – sold as a bulk product) was first evaluated using different concentrations. Considering the initial results (Table 1), the concentration of soybean protein was fixed at 12% (*w*/*w*) per wt of biomass (on a dry basis) in all the subsequent experiments. Bovine serum albumin (BSA) (Sigma, USA) at 12% (*w*/*w*) was used for comparison with the effect of soybean protein.

Pretreated Lignocellulosic Biomass

Steam exploded sugarcane bagasse (SE) was kindly donated by the Sugarcane Research Center (CTC, Brazil). The pretreatment was conducted for 20 min at 1667 kPa and 205 °C. Liquid hot water pretreated bagasse (LHW) was prepared in a 5-L reactor (Model 4580, Parr Instruments) using a 10% (*w*/*v*) solids loading and application of a temperature of 195 °C for 10 min. Heat up and cool down times were 75 and 30 min, respectively. Pretreatment conditions used here were based on previous studies for sugarcane bagasse [23, 27–29]. Neither pretreated material was washed after the pretreatment, and the materials were dried at room temperature. Determination of biomass dry weights was carried out using an infrared moisture analyzer (BEL Engineering, Italy). Chemical characterization of the pretreated bagasses was performed as described previously [30]. The compositions of SE and LHW, respectively, were (*w*/*w*): 41 and 56% glucan; 12 and 6% pentosan; 34 and 29% lignin; and 12 and 4% ash. The two types of pretreated sugarcane bagasse were sieved, and the particle size used was dp ≤ 1 mm.

Sugarcane Bagasse Enzymatic Hydrolysis

Enzymatic hydrolysis of the pretreated sugarcane bagasse (both SE and LHW) was carried out under different conditions in order to evaluate the effect of soybean protein in the saccharification process. The commercial enzyme complex (Cellic Ctec2; 230 FPU/mL) used in the hydrolysis assays was donated by Novozymes Latin America (Araucaria, PR, Brazil). Control experiments employed reaction blanks for both substrates and enzyme loading, in the absence and presence of soybean protein. The glucose released was measured using a D-glucose enzymatic assay kit (Labtest, Brazil), and reducing sugars were determined by the dinitrosalicylic acid (DNS) assay [31]. All the hydrolysis experiments were performed in triplicate, and the data were calculated as means ± standard deviations.

Table 1 Soybean protein concentration (%, *w*/*w*) including the control experiment (absence of soybean protein) and glucose released concentration (g/L) during enzymatic hydrolysis of steam exploded (SE) and liquid hot water-pretreated (LHW) sugarcane bagasses at 10% (*w*/*v*) solids loading and enzyme dosage of 5 FPU/g dry biomass, at 50 °C for 24 h

Soybean protein concentration (%, *w*/*w*)	SE		LHW	
	Glucose released (g/L)	% increase	Glucose released (g/L)	% increase
0	7.20 ± 0.11	–	7.05 ± 0.12	–
4	7.65 ± 0.09	6	8.33 ± 0.18	19
8	8.69 ± 0.19	20	12.44 ± 0.12	77
12	9.20 ± 0.04	28	14.09 ± 0.02	100
20	9.29 ± 0.02	29	14.01 ± 0.09	98

Hybridization Incubator

The hydrolysis experiments were carried out for 24 h in 5 mL tubes placed in a hybridization incubator operated at an agitation speed of 30 rpm. Solids loadings of 10, 15, and 20% (*w*/*v*, dry weight basis) of biomass and enzyme loadings of 5, 10, and 15 FPU/g dry biomass were used, at 50 °C, in sodium citrate buffer (50 mM and pH 4.8). The additives (at the concentrations described in "Additive") were diluted in citrate buffer, before addition of the buffer to the final mixture. For this set of experiments, the conditions were varied one at a time. In addition, a set of experiments to evaluate the temporal profile of enzymatic hydrolysis using the SE and LHW bagasses, in the presence and absence of soybean protein, were conducted up to 96 h of reaction, under the following conditions: solids loading of 15% (*w*/*v*), enzymes loading of 5 FPU/g dry biomass, and 12% (*w*/*w*) of soybean protein. Samples were withdrawn every 24 h for glucose determination.

Stirred Tank Reactor

After the enzymatic hydrolysis conditions had been defined in the tests employing 5 mL tubes, experiments were conducted using a homemade stirred tank reactor. Briefly, the bioreactor had a working volume of 500 mL, internal diameter of 0.085 m, and total height of 0.140 m and was equipped with two aligned three-blade elephant ear (EE) impellers with diameters and spacing between them of 0.040 m and impeller clearance of 0.200 m [32]. Bench-scale saccharification was carried out at 15% (*w*/*v*) biomass loading for both pretreated bagasses, with soybean protein at a concentration of 12% (*w*/w, dry weight basis) and an enzyme dosage of 5 FPU/g dry biomass. The process was performed for 24 h at 50 °C and 400 rpm.

Mechanism of Action of Soybean Protein

Effect of the Additive on Enzyme-Substrate Interactions

Enzymatic saccharification of pure microcrystalline cellulose (Celuflok 200, Celuflok, São Paulo, Brazil) was carried out at 15% (*w*/*v*) solids loading and enzyme dosage of 5 FPU/g biomass, without and with soybean protein or bovine serum albumin (BSA) at a concentration of 12% (*w*/*w*). The experiments were performed for 24 h in 5-mL tubes placed in the hybridization incubator, at 50 °C and 30 rpm. For all the conditions, the glucose concentration was measured using a D-glucose enzymatic assay kit (Labtest, Brazil). All experiments were performed in triplicate, and the data were calculated as means ± standard deviations.

Effect of the Additive on Enzyme Activity

The influence of soybean protein on enzyme activity and stability was investigated by incubating the enzymes with soybean protein and citrate buffer for 24 h, prior to the enzyme assay. The amounts of enzyme, soybean protein, and buffer, as well as the experimental conditions, were the same as for the enzymatic hydrolysis using the hybridization incubator ("Hybridization incubator"). Measurements of endoglucanase, β-glucosidase, and filter paper activity (FPase) were performed using the methodology of [33]. Xylanase activity was determined as described by [34]. Cellobiohydrolase activity was measured using Avicel (microcrystalline cellulose) as the substrate, according to the method described in [35]. For controls, the enzyme was incubated in buffer solution in the absence of soybean protein. All experiments were performed in triplicate, and the data were calculated as means ± standard deviations.

Biomass Characterization after the Adsorption Experiments

Fourier Transform Infrared Spectroscopy Fourier transform infrared spectroscopy (FTIR) analyses were used to characterize the chemical composition of the biomass after incubation for 24 h in the presence of soybean protein. The adsorption experiments employed the hybridization incubator operated under the same conditions used for the enzymatic hydrolysis, with 5 mL tubes containing a final volume of 4 mL. The assays were performed for 24 h, at 30 rpm and 50 °C, with solids loadings of 15% (*w*/*v*) of SE and LHW bagasse, in the presence and absence of soybean

protein at 12% (*w*/w). After 24 h, the samples were filtered under vacuum and the solids were dried at 50 °C for 90 min. A Bruker Vertex 70 instrument fitted with a diamond crystal was operated in attenuated total reflectance (ATR) mode, in the frequency range 4000–400 cm^{-1}, with resolution of 4 cm^{-1} and 32 scans.

Scanning Electron Microscopy The morphologies of the SE and LHW biomasses were analyzed by scanning electron microscopy (SEM) after enzymatic hydrolysis in the presence and absence of soybean protein. The adsorption conditions were the same as those described above for the FTIR analyses. The samples were attached to aluminum stubs with carbon tape and were coated with a layer of gold using a Leica EM SCD050 sputter coater system. Images of the samples were obtained as described by [36], using a JEOL JSM-6510 scanning electron microscope. Isolated soybean protein was also analyzed.

Effect of the Additive on Ethanol Production

The effect of soybean protein on sugar fermentation for ethanol production was evaluated in experiments carried out after the enzymatic hydrolysis of SE and LHW in the presence and absence of the additive. The ethanol fermentations were conducted according to methodology described by [37] and adapted by [38]. Briefly, a commercial strain of *Saccharomyces cerevisiae* yeast (AB Brasil Indústria e Comércio de Alimentos Ltda, Brazil) was used at an initial concentration of 25 g/L (dry basis) [38]. The fermentation medium contained (g/L) KH_2PO_4 (5.6), $MgSO_4{\cdot}7H_2O$ (1.4), yeast extract (6.8), and urea (5.3), at pH 5.0. The initial glucose concentrations used for the control SE and LHW hydrolysates (without soybean protein) were 11.0 ± 0.5 and 13.2 ± 0.6 g/L, respectively. The initial glucose concentrations for the hydrolysates of SE and LHW in the presence of soybean protein were 16.4 ± 1.2 and 23.5 ± 1.2 g/L, respectively. The incubations were performed for 10 h at 200 rpm and 34 °C. Samples were withdrawn every 2 h and were appropriately diluted and filtered through a 22-μm disk filter, prior to analysis. All the experiments were performed in triplicate, and the data were calculated as means ± standard deviations. The concentrations of ethanol (C_E) and glucose (C_G) were determined by high-performance liquid chromatography (HPLC), using a Shimadzu SCL-10A instrument. The conditions and equipment employed for these analyses were the same as those described by [39]. For the ethanol production step (at 8 h), the ethanol yield factor relative to glucose ($Y_{E/G}$, in g_E/g_G) was calculated according to Eq. 1 and was then used to calculate the ethanol yield or efficiency (η_E, in %), relative to the theoretical ethanol yield factor of 0.511 g_E/g_G, according to Eq. 2. The volumetric ethanol productivity (P_E, in g_E/L-h) was calculated using Eq. 3.

$$Y_{E/G} = \frac{C_{E(8\ h)} - C_{E0h}}{C_{G0h} - C_{G(8\ h)}} \quad (1)$$

$$\eta_E = \frac{Y_{E/G}}{0.511} \times 100 \quad (2)$$

$$P_E = \frac{C_{E(8\ h)} - C_{E0h}}{8\ h} \quad (3)$$

Statistical Analysis

Independent replicate data of all enzymatic hydrolysis and ethanol fermentation assays were expressed as means ± standard deviations. Mean comparisons were performed by the Tukey's test ($p < 0.05$) using the software Origin 8.0 (Originlab, USA).

Results and Discussion

Effects of Soybean Protein in the Enzymatic Hydrolysis of Pretreated Sugarcane Bagasse at Different Solids Loadings

Firstly, enzymatic hydrolysis of the two hydrothermally pretreated sugarcane bagasses (SE and LHW) was performed in the presence of different concentrations of soybean protein, using a pretreated bagasse solids loading of 10% (*w*/*v*) and an enzyme dosage of 5 FPU/g dry biomass (Table 1). A positive effect of the use of soybean protein was observed in enzymatic hydrolysis of both pretreated lignocellulosic materials, with the released glucose concentration gradually increasing with increase of the soybean protein concentration up to 12% (*w*/w). Comparing the two types of pretreated bagasse, the most significant positive effect of the addition of soybean protein was found for the enzymatic hydrolysis of LHW, with a 100% increase in glucose released, in the presence of soybean protein at 12% (*w*/*w*). In the case of SE, the increase in glucose released was only up to 28%, using the same soybean protein concentration, when compared to the control experiment without soybean protein. The difference observed between the two hydrothermally pretreated bagasses in the response to the addition of soybean protein could be attributed to the physical and chemical natures of these materials, especially in terms of the type and/or content of lignin, and its properties after the pretreatment process. Although both processes are able to improve the accessibility of enzymes to cellulose, the amounts and characteristics of the resulting fibers can vary [6, 7]. For instance, during the hydrothermal processing, lignin and lignin–hemicellulose linkages can undergo degradation, partial depolymerization, and re-localization [6–9], thus affecting

the degree of unproductive adsorption of enzyme onto the remaining lignin.

Recent studies have concluded that operating the enzymatic hydrolysis process at high-solids concentrations such as 15 or 20% (*w*/*v*) is required in order to ensure economic feasibility at an industrial scale [24, 40]. Therefore, in the next step of the study, an evaluation was conducted using soybean protein (at 12% *w*/*w*) for the enzymatic hydrolysis performed at high-solids loadings (Fig. 1). For the three solids loadings tested (10, 15, and 20% *w*/*v*), there were positive effects of the additive for both SE (Fig. 1a) and LHW (Fig. 1b). Despite the higher glucose concentrations achieved using the higher solids loadings, the increase in the yield of glucose released with the addition of soybean protein was most evident using a solids loading of 10% (*w*/*v*) (Fig. 1c). An improvement of 100% was obtained using LHW pretreated bagasse at 10%, while a 76% improvement was achieved for a solids loading of 15%, which is still very promising. In the case of SE, the improvements in enzymatic hydrolysis performance in the presence of soybean protein were statistically similar (according to Tukey's test) for solids loadings of 10 and 15% (*w*/*v*) (Fig. 1c).

It should be noted that when high-solids loadings are used, the conversion of cellulose is negatively affected by factors such as mass transfer, lignin adsorption, inhibition caused by bioproducts released in the pretreatment, and end-product inhibition by sugars released during the enzymatic hydrolysis process [41]. It is especially likely that mass transfer limitation could have occurred in the enzymatic hydrolysis at 20% (*w*/*v*) solids concentration, due to mixing difficulties caused by the initial viscosity of the material at high-solids loading. On the other hand, the use of high lignocellulosic biomass solids loadings in the enzymatic hydrolysis can be advantageous, due to increase of the final glucose concentration. Therefore, the subsequent experiments were performed using a solids loading of 15% (*w*/*v*) for both types of hydrothermally pretreated sugarcane bagasse.

Effect of Soybean Protein in Enzymatic Hydrolysis Using Different Enzyme Dosages

Enzymatic hydrolyses of the SE and LHW pretreated sugarcane bagasses were performed at 15% (*w*/*v*) solids loadings, in the absence and presence of 12% (*w*/w) soybean protein, using different enzyme dosages (5, 10, and 15 FPU/g dry biomass) (Fig. 2). For both SE (Fig. 2a) and LHW (Fig. 2b), the results showed that for an enzyme dosage of 5 FPU/g biomass with additive, the glucose concentration was equivalent to that obtained using an enzyme dosage of 10 FPU/g biomass in the absence of soybean protein. Therefore, addition of 12% (*w*/*w*) soybean protein enabled the enzyme dosage to be reduced by half, while still achieving similar or even higher saccharification efficiency.

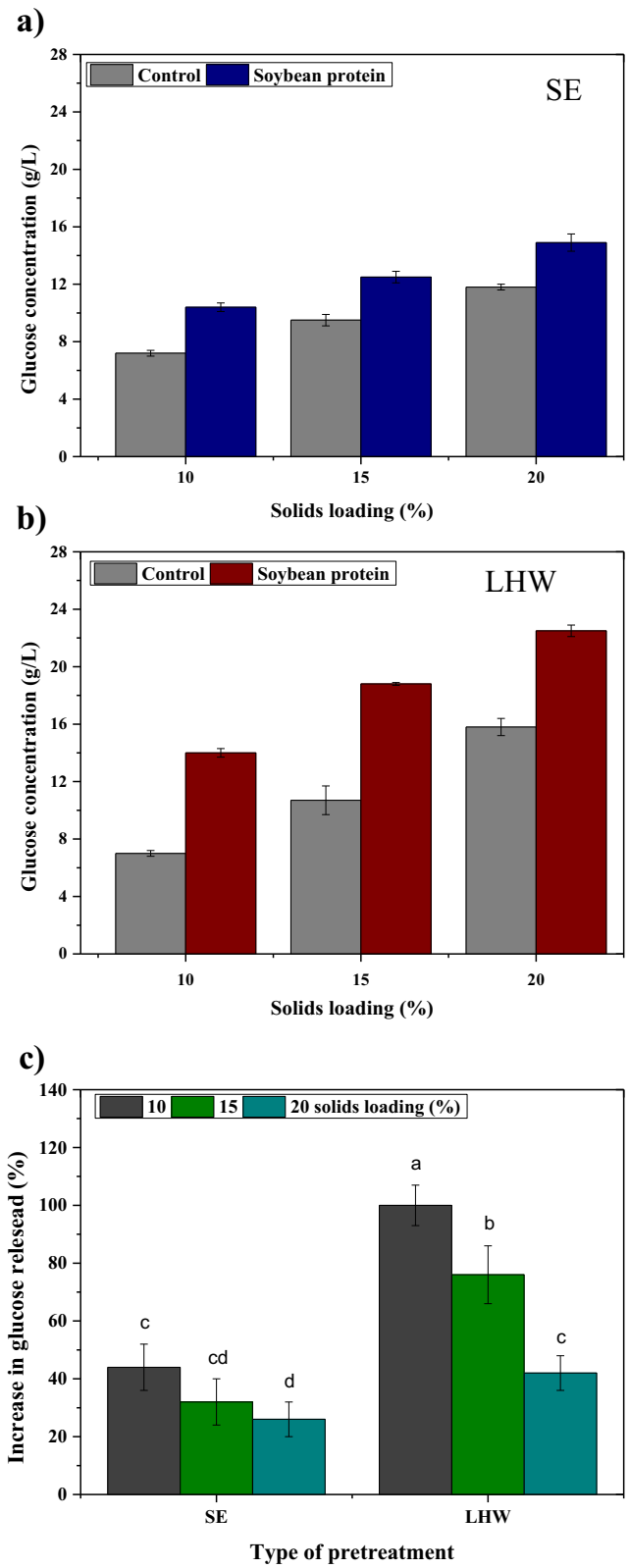

Fig. 1 Effect of soybean protein (12%, *w*/*w*) on enzymatic hydrolysis of **a** SE and **b** LHW with different solids loadings (10, 15, and 20%, *w*/*v*), at 50 °C for 24 h, using commercial enzyme at 5 FPU/g dry biomass, and **c** the increase of glucose released (%) for the two types of pretreated sugarcane bagasse hydrolyzed in the presence of the additive. Different letters indicate a significant difference (Tukey's test, $P < 0.05$)

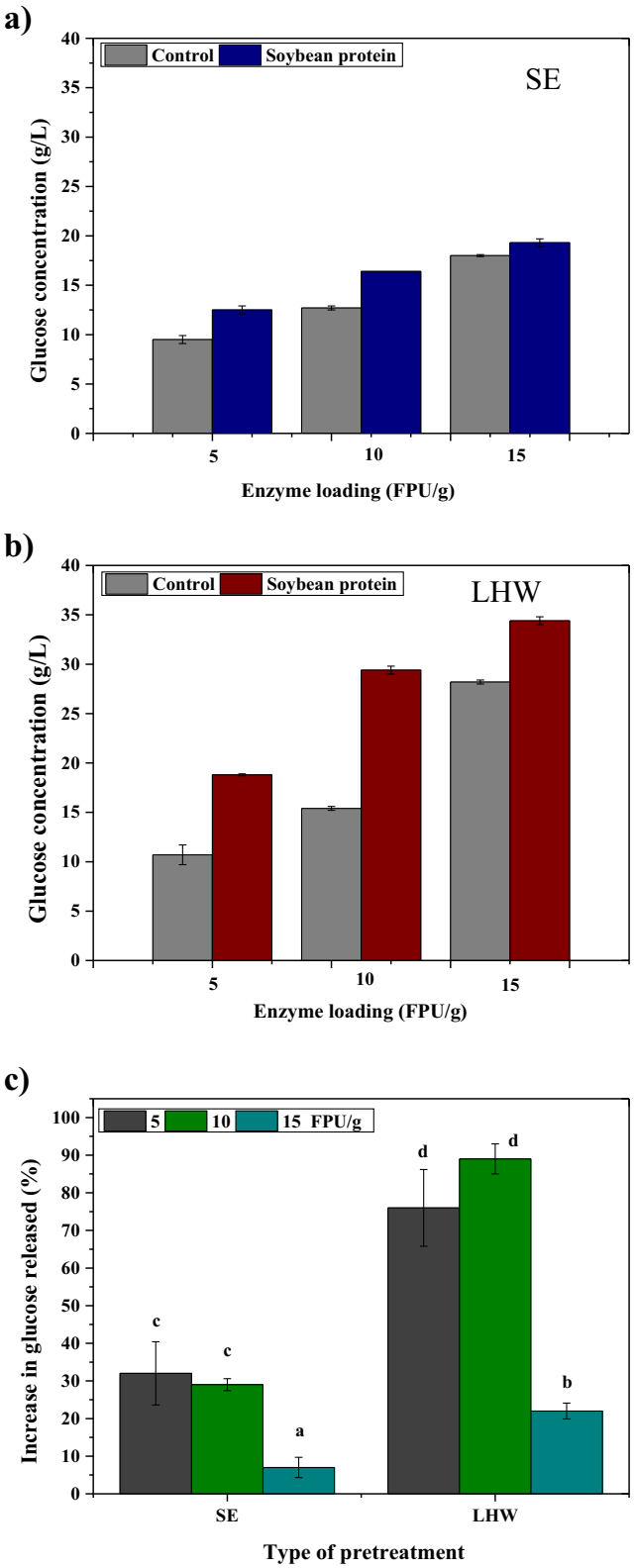

Fig. 2 Effect of soybean protein (12%, *w*/*w*) on enzymatic hydrolysis of **a** SE and **b** LHW with different enzyme dosages (5, 10, and 15 FPU/g dry biomass), using 15% (*w*/*v*) solids loading, and **c** the increase of glucose released (%) for the two pretreated sugarcane bagasses hydrolyzed in the presence of the additive. Different letters indicate a significant difference (Tukey's test, $P < 0.05$)

In terms of percentage, the increases in glucose released using the pretreated bagasses in the presence of soybean protein were greater using smaller enzyme dosages, such as 5 and 10 FPU/g dry biomass (Fig. 2c). Use of these enzyme loadings and addition of soybean protein resulted in significant increases in glucose release for both types of bagasse, compared to the controls. The gains achieved with the use of soybean protein in the enzymatic hydrolysis were similar for these two enzyme dosages (5 and 10 FPU/g biomass), according to Tukey's test, with values of around 30 and 84% for SE and LHW, respectively. As expected, the gain achieved using the soybean protein as an additive decreased as the enzyme loading increased, since the proteins and enzymes present in the cellulolytic enzymatic cocktail itself would act as lignin-blocking proteins. This showed that there was a limit to the improvement in glucose yield due to the addition of soybean protein, with the enhancement being favored by low enzyme loadings, in agreement with previous findings concerning the addition of BSA [42].

In previous studies, saccharification was performed with pretreated wheat straw and different enzyme dosages (5 and 10 FPU/g solids), in the presence of 0.5% (*w*/*w*) fatty alcohol ethoxylate surfactant as an additive. The results showed that the use of surfactant at this concentration enabled the enzyme dosage to be substantially reduced, while maintaining the efficiency of the enzymatic hydrolysis process [43]. Ko et al. [5] used BSA to block the hydrophobic lignin surface of pretreated wood biomass and also found that glucose yields increased in the presence of the additive, at a relatively low enzyme loading of 4.7 mg protein/g biomass. Therefore, the present findings were in agreement with previous studies showing that the use of additives enable the use of low enzyme dosages and improve the performance of saccharification of pretreated lignocellulosic biomass.

Time Profile of Enzymatic Hydrolysis in the Presence of Soybean Protein

In the previous set of experiments, the effect of soybean protein in the enzymatic hydrolysis of SE and LHW was only investigated for a 24-h period. In order to evaluate the action of the additive during a longer period, the time profile of the hydrolysis reaction was followed during 96 h (Fig. 3a). The most evident positive effect of soybean protein addition was observed in the first 24 h for both SE and LHW (Fig. 3b). For the later periods analyzed, the addition of soybean protein had smaller effects on the enzymatic hydrolysis of both pretreated materials. This indicated that the soybean protein had a fairly high affinity for lignin and that the adsorption occurred at the beginning of the reaction, when most of the positive effect was observed.

Similar to these results, the addition of BSA in the enzymatic hydrolysis of pretreated eucalyptus was found to be most effective in the first 24 h [42]. In another study, the effects of the additives BSA and Tween 80 were evaluated

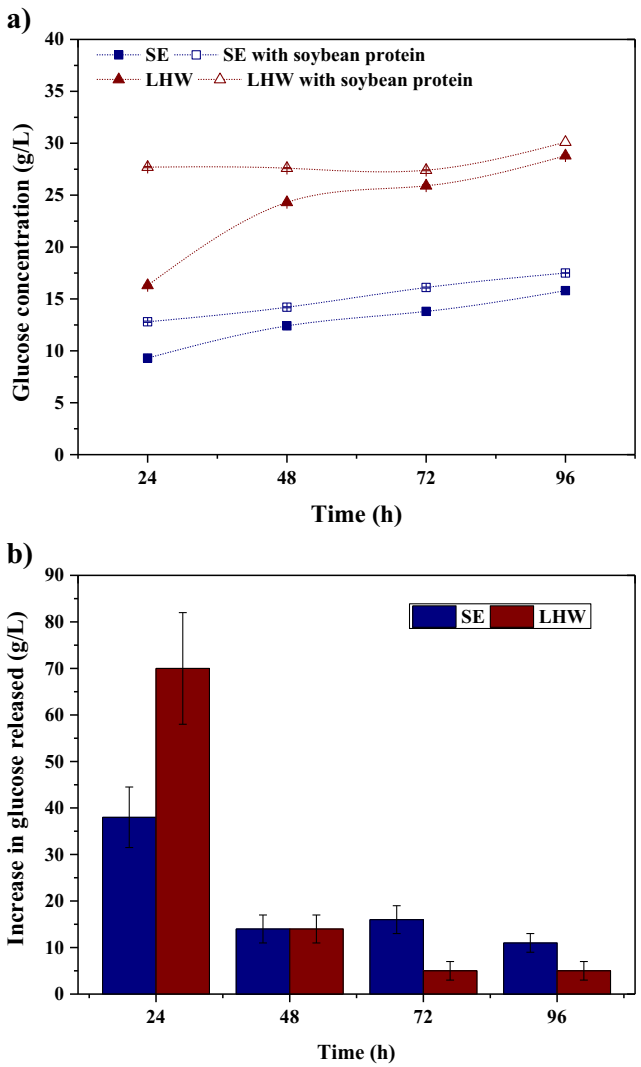

Fig. 3 Effects of soybean protein at 12% (*w*/*w*): **a** on the kinetics of enzymatic hydrolysis of the SE and LHW bagasses for 96 h at 15% (w/v) solids loading and enzyme dosage of 5 FPU/g dry biomass and **b** on glucose release (%), as a function of time, during hydrolysis of the two pretreated sugarcane bagasses

in the enzymatic hydrolysis of pretreated bagasse for 24 and 72 h [44]. It was found that the enhancement of glucose yield with use of the additives decreased as the hydrolysis time was extended from 24 to 72 h, especially for higher enzyme loadings. The results obtained in the present work corroborated the earlier findings that the positive influence of additives occurred mainly in the first 24 h of enzymatic hydrolysis.

Enzymatic Hydrolysis of Pretreated Sugarcane Bagasse with Soybean Protein Using a Bench-Scale Reactor

In order to demonstrate the positive effect of the soybean protein as an additive at higher solids loading, using an agitation system more representative of industrial conditions, the enzymatic hydrolysis of SE and LHW was performed at 15% (*w*/*v*) solids loading in a 0.5-L stirred tank reactor equipped with two three-blade elephant ear (EE) impellers. Experiments carried out in stirred tank reactors provide homogeneity and shear conditions that are more representative of industrial processes [45]. In order to enable comparison, the loadings used were the same as those employed in the flask experiments, with enzyme dosages of 5 FPU/g dry biomass and 12% (*w*/*w*) of soybean protein, in hydrolyses performed for 24 h.

The glucose released during enzymatic hydrolysis of SE and LHW increased by 42 and 61%, compared to enzymatic hydrolysis without soybean protein (Fig. 4). These saccharification efficiency values achieved using the stirred tank reactor were in agreement with the results obtained using shake flasks. However, the conditions of the experiments in the reactor could be further optimized considering parameters such as the agitation velocity and the type of solids feeding (such as fed-batch mode). Adjustment of fed-batch solids feeding conditions has been shown to considerably improve the enzymatic hydrolysis of pretreated sugarcane bagasse in reactors using commercial enzymes [32, 39, 46].

These results confirmed the previous finding that the positive effect of the use of soybean protein as an additive was more significant for LHW, compared to SE. Liquid hot water pretreatment is a hydrothermal treatment that does not require rapid decompression [47], while steam explosion combines mechanical forces and chemical effects, with the biomass being treated with saturated steam at high pressure, followed by reduction of the pressure and an explosive decompression [10, 48]. Therefore, the observed differences could be attributed to the physical and chemical natures of the pretreated materials, especially in terms of the type and/or content of lignin, and its properties after the pretreatment process, as previously observed by [43].

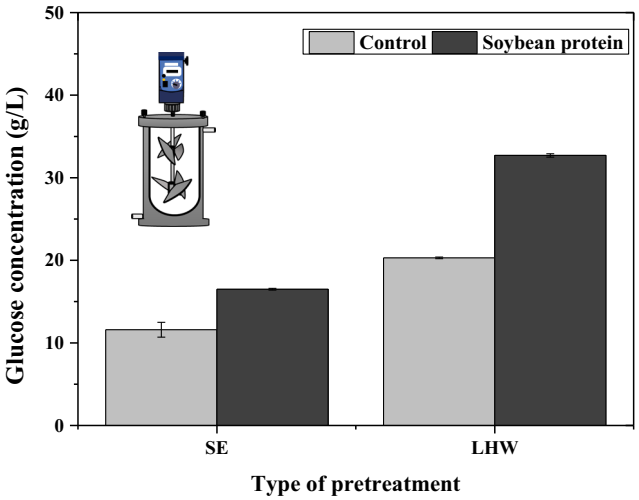

Fig. 4 Effects of soybean protein at 12% (*w*/*w*) on the enzymatic hydrolysis of the SE and LHW bagasses in a bench-scale reactor for 24 h at 500 rpm and 50 °C, using a solids loading of 15% (*w*/*v*) and enzyme dosage of 5 FPU/g dry biomass

In order to further elucidate the mechanism associated with the positive effect of soybean protein during biomass saccharification, three sets of experiments were conducted to elicit (1) the role of soybean protein in enzyme-substrate interaction, (2) the role of soybean protein in the enzymatic activity of cellulases, and (3) the sites for adsorption of soybean protein onto the biomass.

Mechanism of Soybean Protein Action

Effect of the Additive on Enzyme-Substrate Interactions

In order to confirm the role of soybean protein as a lignin-blocking additive, a set of enzymatic hydrolyses was carried out using pure microcrystalline cellulose (Celuflok), which is a substrate that is virtually free of lignin. The Celuflok was hydrolyzed in the absence and presence of soybean protein or BSA at 12% (*w*/*w*). The amounts of glucose released during the enzymatic hydrolysis of Celuflok were similar for the control condition (25.0 ± 1.0 g/L), the use of soybean protein (25.8 ± 0.5 g/L), and the use of BSA (28.5 ± 0.4 g/L). These results indicated that the observed positive effect of soybean protein was probably related to the prevention of unproductive enzyme adsorption, by blocking of the lignin present in the pretreated biomass, as reported previously by [23]. Other studies have reported the positive effect of BSA as a lignin-blocking additive in enzymatic hydrolysis processes [49, 50].

Effect of the Additive on Enzyme Activity

The effects of soybean protein on enzyme activity and stability were evaluated using measurements of EGase, β-glucosidase, FPase, CBH, and xylanase in the absence and presence of the additive (Fig. 5). Enzyme stability was measured by pre-incubating the enzymes and soybean protein for 24 h, prior to the enzyme assay. Enzyme activities were measured at the beginning of the assay, immediately after combining the enzymes with the soybean protein and the substrate. The residual activity was determined as the percentage activity remaining after 24 h. The results showed that the addition of soybean protein led to no improvements in enzyme activity or stability, compared to the control experiments (without soybean protein). The activities of endoglucanase and β-glucosidase remained stable after 24 h of incubation at 50 °C, without and with soybean protein, whereas the activities of FPase, CBH, and xylanase decreased, reaching 50% residual activity for xylanase.

Investigation of the mechanisms according to which additives affect enzyme stability during enzymatic hydrolysis processes has shown that some additives, such as surfactants, act to reduce the contact of enzymes with the air-liquid interface, which could be one of the reasons for improved saccharification of lignocellulosic raw materials [18, 51, 52]. However, the present findings indicated that soybean protein did not improve the activities and stabilities of the enzymes that were evaluated.

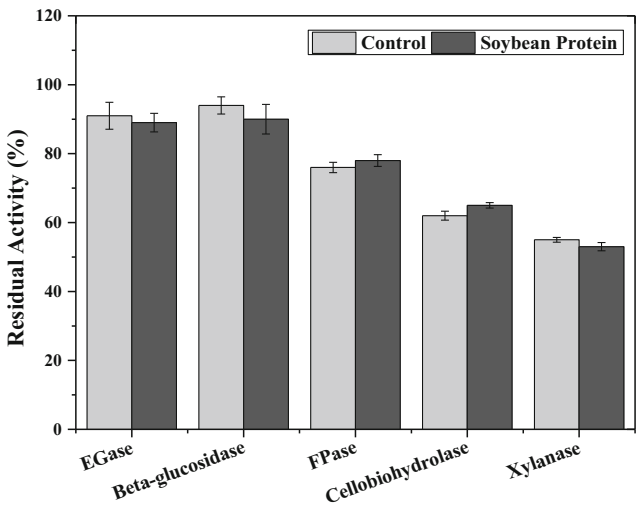

Fig. 5 Effect of addition of soybean protein on the stabilities of EGase, β-glucosidase, FPase, cellobiohydrolase, and xylanase, compared with the control (absence of soybean protein), after 24 h at 50 °C and 30 rpm

Biomass Characterization After the Adsorption Experiments

Comparison was made of the FTIR spectra (Fig. 6) and SEM images (Fig. 7) of the pretreated sugarcane bagasses hydrolyzed in the presence and absence of soybean protein. These experiments were performed in the absence of enzymes. Table 2 highlights some assignments of the signals obtained in the FTIR analysis, especially in the lignin band regions. The discussion of FTIR analysis was based on variations in the relative intensities of component bands [53].

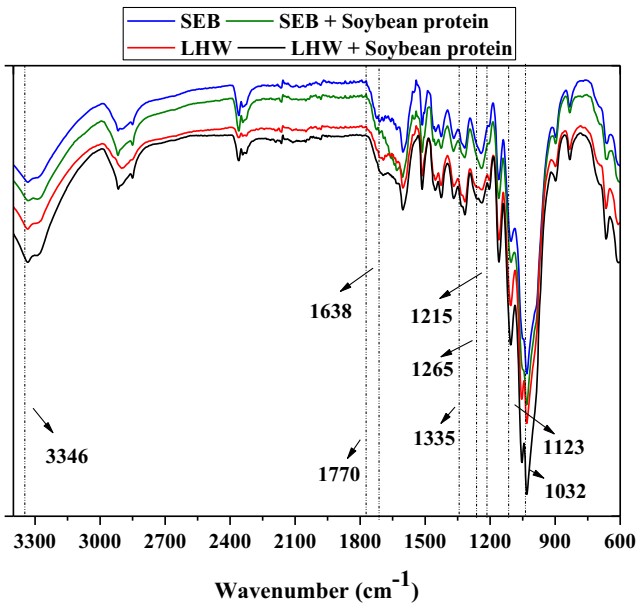

Fig. 6 FTIR spectra of the SE and LHW bagasses after enzymatic hydrolysis in the absence and presence of soybean protein at 12% (*w*/*w*)

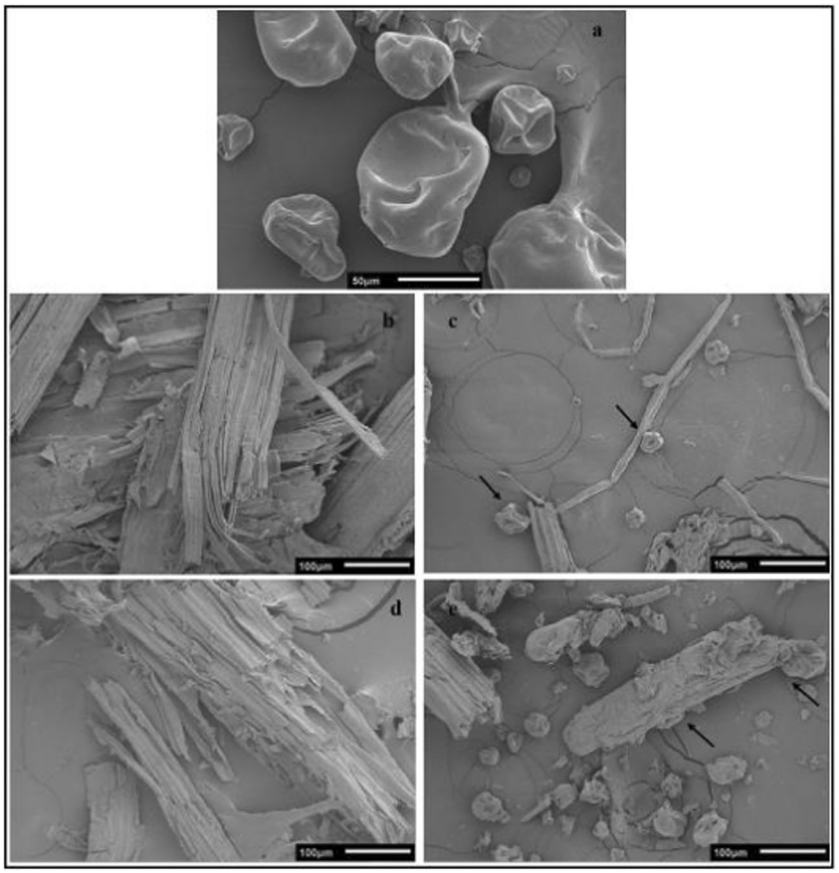

Fig. 7 SEM images. **a** Isolated soybean protein. SE after enzymatic hydrolysis **b** in the absence and **c** in the presence of soybean protein. LHW after enzymatic hydrolysis **d** in the absence and **e** in the presence of soybean protein. The magnification and scale bars are provided in each micrograph

The main features of these spectra could be attributed to the natural components of lignocellulosic biomass: cellulose, hemicellulose, and lignin. The similarity among the spectra indicated that the pretreatment methods applied did not differ greatly in terms of their effects on the structure of the sugarcane bagasse (Fig. 6). However, certain characteristic bands related to the structural components of the biomass can be used to identify structural modification of lignocellulose after conversion processes [54–56]. Bands at 1265 and 1123 cm^{-1} were reported as characteristic of the lignin fraction of sugarcane bagasse [57, 58]. Intense bands for SE and LHW at 1770 and 1638 cm^{-1} (Table 2) corresponded to carbonyl groups whose presence increases the unproductive adsorption of cellulases onto lignin [59]. The intensities of these signals decreased after the addition of soybean protein, possibly due to interaction between the additive and lignin, which would lead to reductions of unproductive enzyme adsorption. The addition of soybean protein appeared to decrease the intensities of bands at 3346 and 1335 cm^{-1}, characteristic of the lignin fraction of sugarcane bagasse [43, 59] and corresponding to O–H stretching

Table 2 Relative band intensities and signal assignments in the FTIR spectra of the SE and LHW bagasses hydrolyzed in the absence and presence of soybean protein

Band positions (cm^{-1})	Assignments	Band intensities			
		SE	SE + soybean protein	LHW	LHW + soybean protein
3346	O–H stretching vibration of OH groups	0.96	0.94	0.99	0.93
1770	Unconjugated carbonyl stretching of lignin	1.05	1.03	1.14	1.08
1638	Conjugated carbonyl stretching of lignin	0.96	0.80	1.14	1.06
1335	R-OH of lignin	0.99	0.98	0.99	0.97
1265	Guaiacyl C–O units	0.99	0.98	1.02	0.98
1215	Guaiacyl ring deformation	1.00	0.99	1.01	0.99
1123	C–H deformation in syringyl units	0.95	0.93	0.98	0.91
1032	C–H deformation in guaiacyl and syringyl units	0.79	0.76	0.87	0.81

vibrations of aromatic and aliphatic –OH groups. These features were suggestive of the interaction of hydrophobic moieties of the soybean protein with hydrophobic groups of the lignin that became exposed after the pretreatment. Bands at 1215 and 1032 cm^{-1}, ascribed to the guaiacyl ring, were more intense for LHW, compared to SE, indicative of greater unproductive adsorption for this type of pretreated bagasse (LHW). It has been reported that lignin with a higher guaiacyl content adsorbs a greater quantity of cellulase enzymes [17]. Here, the presence of soybean protein during the enzymatic hydrolysis resulted in decreased intensities of these bands, for both types of bagasse, which could be another indication of interaction between the additive and the lignin present in the biomass.

Physical insights into the structural changes that occurred during the enzymatic hydrolysis in the presence and absence of soybean protein were obtained using SEM (Fig. 7). Micrographs were acquired at different magnifications, in randomly selected regions of the SE and LHW materials. Representative images are provided in Fig. 7a–e, showing the microstructural and morphological features of isolated soybean protein and the pretreated bagasses after enzymatic hydrolysis in the absence and presence of soybean protein. The surface of the soybean protein was smooth and globular (Fig. 7a). Images of the SE bagasse after enzymatic hydrolysis without and with soybean protein are shown in Fig. 7b, c, respectively. Characteristics of sugar cane bagasse pretreated using steam explosion (Fig. 7b) include reductions of particle size, fiber length, and cellulose crystallinity, while surface area, porosity, and cellulose accessibility are increased [10, 60]. Figure 7c shows soybean protein close to the SE bagasse fibers (indicated with black arrows). The surface of the soybean protein remained globular, but with roughness due to adhered material.

The morphology of the LHW bagasse after enzymatic hydrolysis in the absence and presence of soybean protein is illustrated in Fig. 7d, e. The interfibrillar porosity (indicated by the black arrow in Fig. 7d) was a consequence of partial removal of the hemicelluloses, allowing observation of parenchyma and perforations in the vascular bundles [54, 61]. Condensation reactions of lignin molecules also occur during pretreatment, leading to the formation of spherical lignin droplets (with diameters of ~ 1–10 μm) on the cell wall surface [5]. As in the case of hydrolysis of the SE bagasse (Fig. 7c), soybean protein was observed very close to the fibers, with adhered materials present on the surface (Fig. 7e). The SEM images corroborated the positive results observed when soybean protein was added during the enzymatic hydrolysis.

Effect of the Additive on Ethanol Production

In order to examine the influence of soybean protein in the alcoholic fermentation process, the hydrolysates obtained after saccharification of SE and LHW without and with 12% (*w*/*w*) soybean protein, at 15% (*w*/*v*) solids loading and with an enzyme dosage of 5 FPU/g dry biomass, were fermented by *Saccharomyces cerevisiae* (Fig. 8). The results showed a positive effect of soybean protein on the fermentation efficiency. For the SE bagasse hydrolysates obtained without and with addition of soybean protein, 5.2 ± 0.3 and 8.6 ± 0.1 g/L ethanol were obtained, respectively, after 8 h of fermentation (Fig. 8a), representing an increase of 65% in ethanol production when the soybean protein was used. For LHW, use of the additive resulted in an increase of 86% in ethanol production after 8 h (Fig. 8b), with ethanol values of 6.5 ± 0.2 and 12.1 ± 0.1 g/L for the hydrolysates produced without and with the addition of soybean protein, respectively. These results show that the addition of soybean protein to the saccharification reaction of sugarcane bagasse lead to a higher amount of glucose released, which in turn contributed to increase the production of ethanol as well. Interestingly, the improvement achieved in terms of the ethanol production was higher than the one expected when taking into consideration only the

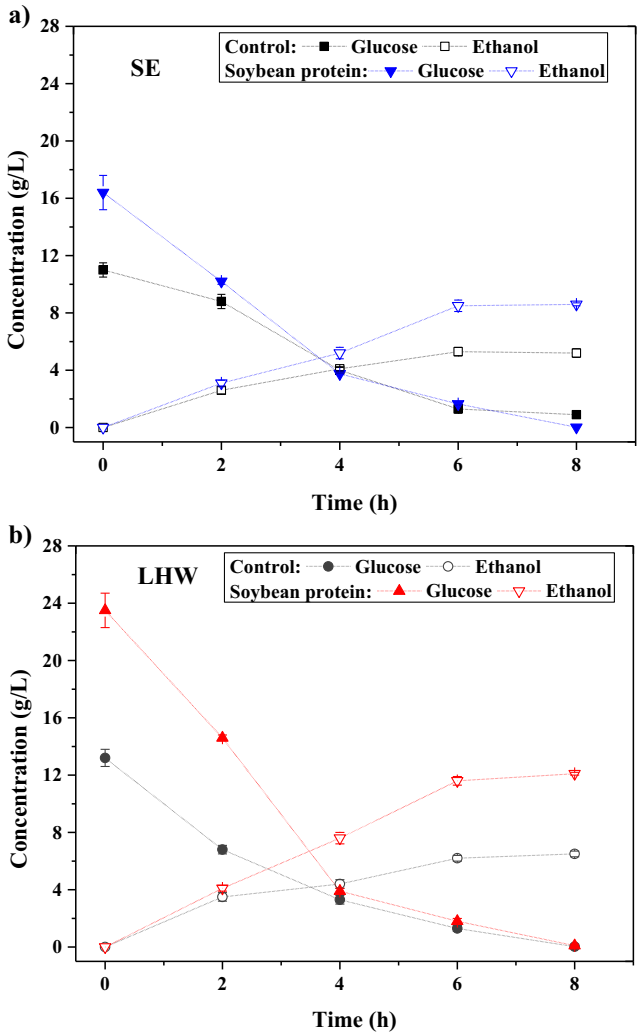

Fig. 8 Ethanol fermentation efficiencies for hydrolysates obtained after saccharification of **a** SE and **b** LHW hydrolyzed in the absence and presence of soybean protein at 15% (*w*/*v*)

difference in the initial glucose concentration values for the conditions with and without the soybean protein. A possible explanation for such favorable effect of soybean protein on ethanol fermentation could be possibly related to the use of the remaining soybean protein in the medium as a nutrient source by the yeast [62, 63]. Another hypothesis is that the soybean protein would be also contributing to minimize the negative effect of yeast fermentation inhibitors that could possibly be present in the biomass hydrolysates.

Determination of the ethanol yield (η_E, as % of the theoretical yield factor) and the volumetric ethanol productivity (P_E, in g/L-h) also provided useful information for evaluation of the processes of ethanol production from the different types of pretreated bagasse hydrolysate obtained in the absence and presence of soybean protein (Table 3). In terms of ethanol yield, no significant differences were observed among the SE and LHW hydrolysates. However, differences in the volumetric ethanol productivity (P_E) were observed between the controls and the SE and LHW hydrolysates produced in the presence of soybean protein. The highest productivities were achieved for the hydrolysates from SE and LHW with soybean protein, with P_E values of 1.1 and 1.5 g/L-h, respectively. Compared to the controls (without soybean protein), the volumetric ethanol productivities were around 65 and 86% higher for the hydrolysates of SE and LHW with soybean protein, respectively. Overall, the results showed that the use of soybean protein had a remarkable positive impact on ethanol fermentation, as well as probably also being used as a nutrient source by the yeast.

In addition to that, it is important to emphasize the relatively lower market price of soybean protein, mainly in comparison to the cellulolytic enzymatic cocktails. In fact, soybean protein is reported to be one of the cheapest proteins available, with a market price of around US$1.25/kg of protein [64]. Furthermore, in biorefineries in which sugarcane and soybean would be used as feedstock to obtain biofuels and other products, the soybean protein would be readily available on-site as a bioproduct. Such approach could potentially contribute to make the application of soybean protein in the lignocellulosic biomass conversion process not only economically feasible but also environmentally beneficial to the overall process, since higher sugar concentrations in the hydrolysis step will lead to reduced energy demands, thus supporting further techno-economic and environmental assessments towards its implementation in a biorefinery context.

Table 3 Ethanol yield (η_E, in % of theoretical yield factor) and volumetric ethanol productivity (P_E, in g/L-h) after 8 h of alcoholic fermentation of pretreated sugarcane bagasses hydrolyzed in the absence and presence of soybean protein

Biomass hydrolysate	η_E (%)	P_E (g/L-h)
SE	93	0.7B
SE + soybean protein	100	1.1A
LHW	96	0.8B
LHW + soybean protein	100	1.5A

Different capital letters indicate significant difference (Tukey's test, $P < 0.05$)

Conclusions

The use of soybean protein as an additive during high-solids processing of hydrothermally pretreated sugarcane bagasse resulted in remarkable improvements in both the enzymatic hydrolysis and fermentation steps. Addition of soybean protein at 12% (*w*/*w*) improved glucose release up to 76% during enzymatic hydrolysis with high-solids loadings (15%, *w*/*v*). The apparent mechanism of action of the soybean protein was that it functioned as a lignin-blocking additive in the enzymatic hydrolysis. Furthermore, the presence of soybean protein increased the production of ethanol from the SE and LHW hydrolysates by 65 and 86%, respectively. These findings are relevant for the development of lignocellulosic ethanol processes, since soybean protein can be used as a cost-effective alternative additive that benefits the enzymatic hydrolysis and alcoholic fermentation steps.

Acknowledgements We are grateful to Dr. Eduardo Ximenes and Dr. Michael Ladisch from Purdue University (IN, USA) for their very insightful suggestions for this work.

Funding information Embrapa, CNPq (Process 401182/2014-2), CAPES, and FAPESP (Processes 2014/19000-3 and 2016/10636-8) (all from Brazil) provided financial support.

References

1. Lynd LR, Liang XY, Biddy MJ, Allee A, Cai H, Foust T, Himmel ME, Laser MS, Wang M, Wyman CE (2017) Cellulosic ethanol: status and innovation. Curr Opin Biotechnol 45:202–211
2. Kumar R, Wyman CE (2009) Access of cellulase to cellulose and lignin for poplar solids produced by leading pretreatment technologies. Biotechnol Prog 25:807–819
3. Lu X, Wang C, Li X, Zhao J (2017) Temperature and pH influence adsorption of cellobiohydrolase onto lignin by changing the protein properties. Bioresour Technol 245:819–825
4. Tang Y, Chandra RP, Sokhansanj S, Saddler JN (2018) Influence of steam explosion processes on the durability and enzymatic digestibility of wood pellets. Fuel 211:87–94
5. Ko JK, Kim Y, Ximenes E, Ladisch MR (2015) Effect of liquid hot water pretreatment severity on properties of hardwood lignin and enzymatic hydrolysis of cellulose. Biotechnol Bioeng 112:252–262
6. Ruiz HA, Rodriguez-Jasso RM, Fernandes BD, Vicente AA, Teixeira JA (2013) Hydrothermal processing, as an alternative for upgrading agriculture residues and marine biomass according to the biorefinery concept: a review. Renew Sustain Energy Rev 21:35–51
7. Tekin K, Karagoz S, Bektas S (2014) A review of hydrothermal biomass processing. Renew Sustain Energy Rev 40:673–687

8. Mosier N, Hendrickson R, Ho N, Sedlak M, Ladisch MR (2005) Optimization of pH controlled liquid hot water pretreatment of corn stover. Bioresour Technol 96:1986–1993
9. Garrote G, Dominguez H, Parajo JC (1999) Hydrothermal processing of lignocellulosic materials. Holz Als Roh-Und Werkstoff 57: 191–202
10. Arenas-Cardenas P, Lopez-Lopez A, Moeller-Chavez GE, Leon-Becerril E (2017) Current pretreatments of lignocellulosic residues in the production of bioethanol. Waste Biomass Valoriz 8:161–181
11. Kim Y, Kreke T, Hendrickson R, Parenti J, Ladisch MR (2013) Fractionation of cellulase and fermentation inhibitors from steam pretreated mixed hardwood. Bioresour Technol 135:30–38
12. Ximenes E, Kim Y, Mosier N, Dien B, Ladisch M (2010) Inhibition of cellulases by phenols. Enzym Microb Technol 46:170–176
13. Ximenes E, Kim Y, Mosier N, Dien B, Ladisch M (2011) Deactivation of cellulases by phenols. Enzym Microb Technol 48:54–60
14. Kim Y, Ximenes E, Mosier NS, Ladisch MR (2011) Soluble inhibitors/deactivators of cellulase enzymes from lignocellulosic biomass. Enzym Microb Technol 48:408–415
15. Qin L, Li WC, Liu L, Zhu JQ, Li X, Li BZ, Yuan YJ (2016) Inhibition of lignin-derived phenolic compounds to cellulase. Biotechnol Biofuels 9:10
16. Kim Y, Kreke T, Ko JK, Ladisch MR (2015) Hydrolysis-determining substrate characteristics in liquid hot water pretreated hardwood. Biotechnol Bioeng 112:677–687
17. Ko JK, Ximenes E, Kim Y, Ladisch MR (2015) Adsorption of enzyme onto lignins of liquid hot water pretreated hardwoods. Biotechnol Bioeng 112:447–456
18. Eriksson T, Borjesson J, Tjerneld F (2002) Mechanism of surfactant effect in enzymatic hydrolysis of lignocellulose. Enzym Microb Technol 31:353–364
19. Lee D, Yu AHC, Saddler JN (1995) Evaluation of cellulase recycling strategies for the hydrolysis of lignocellulosic substrates. Biotechnol Bioeng 45:328–336
20. Yang B, Wyman CE (2006) Lignin blockers and uses thereof. US Patent
21. Yang B, Wyman CE (2006) BSA treatment to enhance enzymatic hydrolysis of cellulose in lignin containing substrates. Biotechnol Bioeng 94:611–617
22. Kumar R, Wyman CE (2009) Effect of additives on the digestibility of corn Stover solids following pretreatment by leading technologies. Biotechnol Bioeng 102:1544–1557
23. Florencio C, Badino AC, Farinas CS (2016) Soybean protein as a cost-effective lignin-blocking additive for the saccharification of sugarcane bagasse. Bioresour Technol 221:172–180
24. Cannella D, Jorgensen H (2014) Do new cellulolytic enzyme preparations affect the industrial strategies for high solids lignocellulosic ethanol production? Biotechnol Bioeng 111:59–68
25. Jin WX, Chen L, Hu M, Sun D, Li A, Li Y, Hu Z, Zhou SG, Tu YY, Xia T, Wang YT, Xie GS, Li YB, Bai BW, Peng LC (2016) Tween-80 is effective for enhancing steam-exploded biomass enzymatic saccharification and ethanol production by specifically lessening cellulase absorption with lignin in common reed. Appl Energy 175:82–90
26. Brondi MG, Vasconcellos VM, Giordano RC, Farinas CS (2018) Alternative low-cost additives to improve the saccharification of lignocellulosic biomass. Appl Biochem Biotechnol. https://doi.org/10.1007/s12010-018-2834-z
27. Carrasco C, Baudel HM, Sendelius J, Modig T, Roslander C, Galbe M, Hahn-Hagerdal B, Zacchi G, Liden G (2010) SO2-catalyzed steam pretreatment and fermentation of enzymatically hydrolyzed sugarcane bagasse. Enzym Microb Technol 46:64–73
28. Cunha FM, Badino AC, Farinas CS (2017) Effect of a novel method for in-house cellulase production on 2G ethanol yields. Biocatal Agric Biotechnol 9:224–229
29. Rocha GJM, Silva VFN, Martin C, Goncalves AR, Nascimento VM, Souto-Maior AM (2013) Effect of xylan and lignin removal by hydrothermal pretreatment on enzymatic conversion of sugarcane bagasse cellulose for second generation ethanol production. Sugar Tech 15:390–398
30. Gouveia ER, do Nascimento RT, Souto-Maior AM, Moraes Rocha GJ (2009) Validation of methodology for the chemical characterization of sugar cane bagasse. Quim Nova 32:1500–1503
31. Miller GL (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem 31:426–428
32. Squinca P, Badino AC, Farinas CS (2018) A closed-loop strategy for endoglucanase production using sugarcane bagasse liquefied by a home-made enzymatic cocktail. Bioresour Technol 249:976–982
33. Ghose TK (1987) Measurement of cellulase activities. Pure Appl Chem 59:257–268
34. Bailey MJ, Poutanen K (1989) Production of xylanolytic enzymes by strains of *Aspergillus*. Appl Microbiol Biotechnol 30:5–10
35. Florencio C, Cunha FM, Badino AC, Farinas CS (2015) Validation of a novel sequential cultivation method for the production of enzymatic cocktails from *Trichoderma* strains. Appl Biochem Biotechnol 175:1389–1402
36. Pereira SC, Maehara L, Machado CMM, Farinas CS (2016) Physical-chemical-morphological characterization of the whole sugarcane lignocellulosic biomass used for 2G ethanol production by spectroscopy and microscopy techniques. Renew Energy 87: 607–617
37. Sonego JLS, Lemos DA, Rodriguez GY, Cruz AJG, Badino AC (2014) Extractive batch fermentation with CO2 stripping for ethanol production in a bubble column bioreactor: experimental and modeling. Energy Fuel 28:7552–7559
38. Bondancia TJ, Mattoso LHC, Marconcini JM, Farinas CS (2017) A new approach to obtain cellulose nanocrystals and ethanol from Eucalyptus cellulose pulp via the biochemical pathway. Biotechnol Prog 33:1085–1095
39. Correa LJ, Badino AC, Cruz AJG (2016) Power consumption evaluation of different fed-batch strategies for enzymatic hydrolysis of sugarcane bagasse. Bioprocess Biosyst Eng 39:825–833
40. Modenbach AA, Nokes SE (2013) Enzymatic hydrolysis of biomass at high-solids loadings—a review. Biomass Bioenergy 56: 526–544
41. Du J, Li Y, Zhang H, Zheng H, Huang H (2014) Factors to decrease the cellulose conversion of enzymatic hydrolysis of lignocellulose at high solid concentrations. Cellulose 21:2409–2417
42. Wei WQ, Wu SB (2017) Enhanced enzymatic hydrolysis of eucalyptus by synergy of zinc chloride hydrate pretreatment and bovine serum albumin. Bioresour Technol 245:289–295
43. Agrawal R, Satlewal A, Kapoor M, Mondal S, Basu B (2017) Investigating the enzyme-lignin binding with surfactants for improved saccharification of pilot scale pretreated wheat straw. Bioresour Technol 224:411–418
44. Zhang HD, Ye GY, Wei YT, Li X, Zhang AP, Xie J (2017) Enhanced enzymatic hydrolysis of sugarcane bagasse with ferric chloride pretreatment and surfactant. Bioresour Technol 229:96–103
45. Buffo MM, Correa LJ, Esperanca MN, Cruz AJG, Farinas CS, Badino AC (2016) Influence of dual-impeller type and configuration on oxygen transfer,. Power consumption, and shear rate in a stirred tank bioreactor. Biochem Eng J 114:133–142
46. Correa LJ, Badino AC, Goncalves Cruz AJ (2016) Mixing design for enzymatic hydrolysis of sugarcane bagasse: methodology for selection of impeller configuration. Bioprocess Biosyst Eng 39: 285–294
47. Alvira P, Tomas-Pejo E, Ballesteros M, Negro MJ (2010) Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: a review. Bioresour Technol 101:4851–4861

48. Carvalheiro F, Duarte LC, Girio FM (2008) Hemicellulose biorefineries: a review on biomass pretreatments. J Sci Ind Res 67:849–864
49. Bhagia S, Kumar R, Wyman CE (2017) Effects of dilute acid and flowthrough pretreatments and BSA supplementation on enzymatic deconstruction of poplar by cellulase and xylanase. Carbohydr Polym 157:1940–1948
50. Brethauer S, Studer MH, Yang B, Wyman CE (2011) The effect of bovine serum albumin on batch and continuous enzymatic cellulose hydrolysis mixed by stirring or shaking. Bioresour Technol 102: 6295–6298
51. Hsieh C-w C, Cannella D, Jorgensen H, Felby C, Thygesen LG (2015) Cellobiohydrolase and endoglucanase respond differently to surfactants during the hydrolysis of cellulose. Biotechnol Biofuels 8:52
52. Yang M, Zhang JH, Kuittinen S, Vepsalainen J, Soininen P, Keinanen M, Pappinen A (2015) Enhanced sugar production from pretreated barley straw by additive xylanase and surfactants in enzymatic hydrolysis for acetone-butanol-ethanol fermentation. Bioresour Technol 189:131–137
53. Jackson M, Mantsch HH (1995) The use and misuse of FTIR spectroscopy in the determination of protein structure. Crit Rev Biochem Mol Biol 30:95–120
54. Rodriguez-Zuniga UF, Neto VB, Couri S, Crestana S, Farinas CS (2014) Use of spectroscopic and imaging techniques to evaluate pretreated sugarcane bagasse as a substrate for cellulase production under solid-state fermentation. Appl Biochem Biotechnol 172: 2348–2362
55. Kristensen JB, Thygesen LG, Felby C, Jorgensen H, Elder T (2008) Cell-wall structural changes in wheat straw pretreated for bioethanol production. Biotechnol Biofuels 1:5
56. Schwanninger M, Rodrigues JC, Pereira H, Hinterstoisser B (2004) Effects of short-time vibratory ball milling on the shape of FT-IR spectra of wood and cellulose. Vib Spectrosc 36:23–40
57. Qi GX, Peng F, Xiong L, Lin XQ, Huang C, Li HL, Chen XF, Chen XD (2017) Extraction and characterization of wax from sugarcane bagasse and the enzymatic hydrolysis of dewaxed sugarcane bagasse. Prep Biochem Biotechnol 47:276–281
58. Camargo LA, Pereira SC, Correa AC, Farinas CS, Marconcini JM, Mattoso LHC (2016) Feasibility of manufacturing cellulose nanocrystals from the solid residues of second-generation ethanol production from sugarcane bagasse. Bioenergy Res 9:894–906
59. Lu XQ, Zheng XJ, Li XZ, Zhao J (2016) Adsorption and mechanism of cellulase enzymes onto lignin isolated from corn stover pretreated with liquid hot water. Biotechnol Biofuels 9:118
60. Novaes Reis Corrales RC, Teixeira Mendes FM, Perrone CC, Sant'Anna C, de Souza W, Abud Y, da Silva Bon EP, Ferreira-Leitao V (2012) Structural evaluation of sugar cane bagasse steam pretreated in the presence of CO2 and SO2. Biotechnol Biofuels 5: 36. https://doi.org/10.1186/1754-6834-5-36
61. Xu Z, Wang Q, Jiang Z, Yang X-x, Ji Y (2007) Enzymatic hydrolysis of pretreated soybean straw. Biomass Bioenergy 31:162–167
62. Crepin L, Truong NM, Bloem A, Sanchez I, Dequin S, Camarasa C (2017) Management of multiple nitrogen sources during wine fermentation by Saccharomyces cerevisiae. Appl Environ Microbiol 83(5):e02617-16
63. Lekkas C, Stewart GG, Hill AE, Taidi B, Hodgson J (2012) Elucidation of the role of nitrogenous wort components in yeast fermentation. J Inst Brew 113:3–8
64. Klein-Marcuschamer D, Oleskowicz-Popiel P, Simmons BA, Blanch HW (2012) The challenge of enzyme cost in the production of lignocellulosic biofuels. Biotechnol Bioeng 109:1083–1087