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Production of cellulosic ethanol from steam-exploded *Eucalyptus urograndis* and sugarcane bagasse at high total solids and low enzyme loadings

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Abstract

Cellulosic ethanol is one of the most important biotechnological products to mitigate the consumption of fossil fuels and to increase the use of renewable resources for fuels and chemicals. By performing this process at high total solids (TS) and low enzyme loadings (EL), one can achieve significant improvements in the overall cellulosic ethanol production process. In this work, steam-exploded materials were obtained from *Eucalyptus urograndis* chips and sugarcane bagasse to be subsequently used for enzymatic hydrolysis at high TS (20 wt%) and relatively low EL (13.3 FPU g^{-1} TS of Cellic CTec3 from Novozymes). Also, the fermentability of their corresponding hydrolysates was tested using an industrial strain of *Saccharomyces cerevisiae* (Thermosacc Dry from Lallemand). Enzymatic hydrolysis of steam-treated *E. urograndis* reached 125 g L^{-1} of glucose in 72 h, while steam-treated bagasse gave yields 25 % lower. Both substrate hydrolysates were easily converted to ethanol, giving yields above 25 g L^{-1} and productivities of 2.3 g L^{-1} h^{-1} for eucalypt and 2.2 g L^{-1} h^{-1} for bagasse after only 12 h of fermentation. Under the conditions used in this study, sugarcane bagasse glucans showed the potential to boost the ethanol production from sugarcane culms by 31 %, from the 80 L t^{-1} of first generation to a total production of 105 L t^{-1}. On the other hand, *E. urograndis* plantations are able to achieve cellulosic ethanol productivities of 2832.2 L ha^{-1} $year^{-1}$, which was 57.8 % higher than the projected value of 1794.5 L ha^{-1} $year^{-1}$ that was obtained for sugarcane bagasse.

Keywords: Steam explosion, Sugarcane bagasse, *Eucalyptus urograndis*, Cellic CTec3, Thermosacc dry, Cellulosic ethanol

Background

Cellulosic ethanol is currently produced by fermentation of carbohydrates that are released from plant polysaccharides by enzymatic hydrolysis. Such production process depends on five sequential steps involving: (1) collection and preparation of plant biomass; (2) pretreatment for increasing the susceptibility of plant polysaccharides to bioconversion at high process yields; (3) enzymatic hydrolysis for converting plant polysaccharides into fermentable sugars; (4) microbial fermentation for cellulosic ethanol production; and, finally, (5) the recovery of ethanol by distillation [1–3].

Several agro-industrial and forestry residues are of great interest as feedstocks for cellulosic ethanol production, such as in the case of sugarcane bagasse and short rotation clones of *Eucalyptus* sp., respectively [4]. Brazil produced 659 million tons of sugarcane in the 2014/2015 harvest season and this resulted in the accumulation of 92 million tons of sugarcane bagasse (14 % on dry basis) after its processing for sucrose and/or first generation ethanol [5, 6]. Most of this bagasse is currently used for energy purposes but there is a surplus that still represents a great opportunity for the development of sustainable biorefineries. On the other hand, over 55 % of the world

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eucalypt production is located in South America [7] and Brazil has an important role as a world leader in the production of short fibers for pulp and paper, mostly from *Eucalyptus urograndis*, *E. saligna* and *E. urophylla* [8]. In addition, other species of *Eucalyptus* sp. and some of their hybrids have shown great potential for the development of bioenergy projects such as clones of *E. urograndis* that have fast growth rates and high wood density [9, 10]. Hence, the availability and favorable properties of these lignocellulosic materials has motivated a great interest in their use for the production of cellulosic ethanol and other important building blocks for the chemical industry, as well as composites and structural materials in both nano and fiber scales [11–13]. Besides, biomass upgrading to fuels, chemicals and materials offers no immediate risks to food security issues.

Steam explosion is one of the most widely used pretreatment method for cellulosic ethanol production [14–16]. This method increases the accessibility of cellulose by deconstructing the associative structure of the plant cell wall, causing an increase in the substrate surface area and pore volume. Changes are also introduced into the biomass chemistry, particularly by acid hydrolysis and partial dissolution of hemicelluloses and lignin [17, 18]. However, pretreatment conditions must be optimized to avoid undesirable side reactions such as carbohydrate dehydration and lignin condensation.

Enzymatic hydrolysis is accomplished by different classes of enzymes which are intended to convert all of the available plant polysaccharides into fermentable sugars. According to Wingren et al. [19], an increase of substrate total solids (TS) from 5 to 8 wt% can reduce the cellulosic ethanol production cost by 20 %. Hence, by performing this step at high TS and low enzyme loadings (EL), it is possible to achieve significant improvements in the economics of the overall cellulosic ethanol production process. These improvements involve a better integration of process streams, energy savings and reductions in capital cost for hydrolysis and distillation, particularly coming from the fact that high sugar concentrations are produced for fermentation [2, 20–22].

Different process configurations have been developed so far for cellulosic ethanol production. If fermentation is carried out after enzymatic hydrolysis using substrate hydrolysates that were filtered to remove suspended solids, the process configuration is referred to as separate hydrolysis and fermentation (SHF). The advantages of SHF are that both steps can be carried out at their optimal conditions and yeast recycling is perfectly feasible, but the capital cost is higher and enzymatic hydrolysis is limited by end-product inhibition. Ethanol can also be produced by simultaneous saccharification and fermentation

(SSF) and, in this case, the capital cost is lower, the total processing time is reduced and end-product inhibition is alleviated because sugars are fermented as they are produced by the concerted action of the enzymes. However, SSF does not allow yeast recycling and hydrolysis is usually carried out below its optimal temperature. In both configurations mentioned above, ethanol yields can be increased if the fermenting organism is able to convert pentoses and hexoses simultaneously [23, 24].

This work compares the efficiency of steam explosion in producing cellulosic ethanol at high TS and relatively low EL from two different lignocellulosic materials. Wood chips of *E. urograndis* and sugarcane bagasse were used for this purpose and ethanol was produced by SHF because we aimed to assess cellulose accessibility without the inhibitory effect of pretreatment water solubles.

Results and discussion
Chemical analysis and pretreatment by steam explosion
As expected, differences were observed in the chemical composition of both native sugarcane bagasse and *E. urograndis* chips (Table 1) and these were in good agreement with the general knowledge about the compositional analysis of both grasses [16, 17, 25] and hardwoods [5, 26]. In general, sugarcane bagasse presented lower glucan and lignin contents while its total extractives, pentosan and ash contents were much higher than those of *E. urograndis* wood chips. Both eucalypt and sugarcane bagasse hemicelluloses were partially quantified as xylans because the High Performance Liquid Chromatography (HPLC) method used for analysis was not able to quantify uronic acids and to resolve xylose, galactose and mannose in biomass acid hydrolysates. The extractives content was generally higher for sugarcane bagasse due to the presence of larger quantities of waxes, cinnamic acid derivatives, terpenes, lipids and other extractable materials [25, 27]. On the other hand, the high ash content of sugarcane bagasse may be partially due to unavoidable contaminations with soil, which are due to the current mechanical harvesting techniques. Biogenic ashes usually contain variable amounts of alkaline and alkaline earth oxides that are easily leached out by a mild acid treatment. Hence, the buffering capacity of this acid leaching may partially compromise the efficiency of pretreatment methods that rely on acid hydrolysis. However, the buffering capacity of sugarcane bagasse is not very high because basic oxides do not account for more than 7 % of its total ash content [25].

The pretreatment mass recovery yields were similar for both lignocellulosic materials, being 64 wt% for water-insoluble steam-treated sugarcane bagasse (STB-WI) and 61.5 wt% for water-insoluble steam-treated eucalypt

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chips (STE-WI), even though different severity factors (log R_O) of 3.67 and 3.94 were applied in each case, respectively.

The chemical composition of STB-WI contained 46.50 % glucans and 2.74 % xylans, whereas STE-WI had 56.34 % glucans and only 1.07 % xylans, with acetic acid being undetectable in both substrate acid hydrolysates (Table 1). The ash content of STB-WI was higher than that of sugarcane bagasse and this was probably a result of its abrasiveness when processed at high total solids. STB-WI had a higher acid-insoluble lignin content compared to STE-WI as well as a higher amount of dehydrated pentoses, which were attributed to the presence of arabinoxylans in sugarcane bagasse. Also, the amount of dehydrated pentoses in steam-treated substrates was much lower than that of native materials, with hydroxymethylfurfural becoming the main sugar dehydration by-product. This observation was not only associated to the lower hemicellulose content of steam-treated substrates but also to the higher chemical accessibility of their steam-treated glucans.

Aguiar et al. [28] steam-treated sugarcane bagasse at 210 °C for 4 min for a severity factor (log R_O) of 3.84. The chemical composition of the resulting material showed 56.6, 5.3 and 33.0 % of glucans, pentosans and total lignin, respectively. Compared to Table 1, it is clear that the use of higher severity factors led to a more extensive removal of the sugarcane bagasse hemicellulose component. On the other hand, Martin-Sampedro et al. [29] steam-exploded *E. globulus* chips at various pretreatment severities and produced substrates with relatively high hemicellulose contents. For instance, substrates containing 53.4 % glucan, 12.7 % xylan and 21.9 % lignin were obtained by steam explosion at a log R_O of 3.35. Such xylan content is much higher than that of STE-WI in Table 1 and this was an effect of pretreatment at a lower severity factor. However, differences in pretreatment procedure as well as in the chemical composition of both eucalypt species may have been highly influential as well.

Enzymatic hydrolysis

The Fig. 1 shows the logarithmic profile of glucose release that was obtained after 72 h enzymatic hydrolysis of both pretreated materials with 62.5 mg of Cellic CTec3 g^{-1} TS, which is equivalent to 13.3 FPU g^{-1} TS. Despite their similar enzymatic hydrolysis profile, conversion of cellulose to soluble sugars was higher for eucalypt compared to bagasse. The final concentrations in glucose equivalents (GlcEq) was 103 g L^{-1} for STB-WI and 125 g L^{-1} for STE-WI and these conversion values were approximately 95 % of the available glucans in both cases, with cellobiose being always a minor component of the substrate hydrolysates (less than 1 %).

Table 1 Chemical compositions of native and pretreated sugarcane bagasse and *E. urograndis*

Components	Sugarcane bagasse		E. urograndis	
	Native	Pretreated	Native	Pretreated
Anhydroglucose	36.32 ± 0.77	46.50 ± 3.12	44.72 ± 0.78	56.34 ± 1.36
Anhydroxylose	15.62 ± 1.51	2.74 ± 0.44	12.25 ± 0.21	1.07 ± 0.03
Anhydroarabinose	1.40 ± 0.59	bdl	bdl	bdl
Acetyl group	2.58 ± 0.29	bdl	2.94 ± 0.22	bdl
Unidentified anhydrohexose[a]	1.91 ± 0.03	2.24 ± 0.01	2.13 ± 0.03	2.10 ± 0.02
Unidentified anhydropentose[b]	6.40 ± 1.35	0.95 ± 0.09	3.11 ± 0.77	0.92 ± 0.44
Acid soluble lignin	0.06 ± 0.01	0.04 ± 0.01	0.06 ± 0.01	0.04 ± 0.09
Acid insoluble lignin	22.13 ± 1.63	36.66 ± 1.18	25.16 ± 1.40	39.06 ± 1.86
Total extractives	5.34 ± 0.49	nd	1.72 ± 0.11	nd
Ashes	7.79 ± 0.53	10.43 ± 0.65	0.36 ± 0.06	0.32 ± 0.03
Total	99.55	99.56	99.83	99.85
Total glucan content[c]	38.23	48.74	46.85	58.44
Total hemicellulose content[d]	26.00	3.69	18.30	1.99
Total lignin content[e]	22.19	36.70	25.22	39.10

nd not detected; *bld* below the detection level of the method
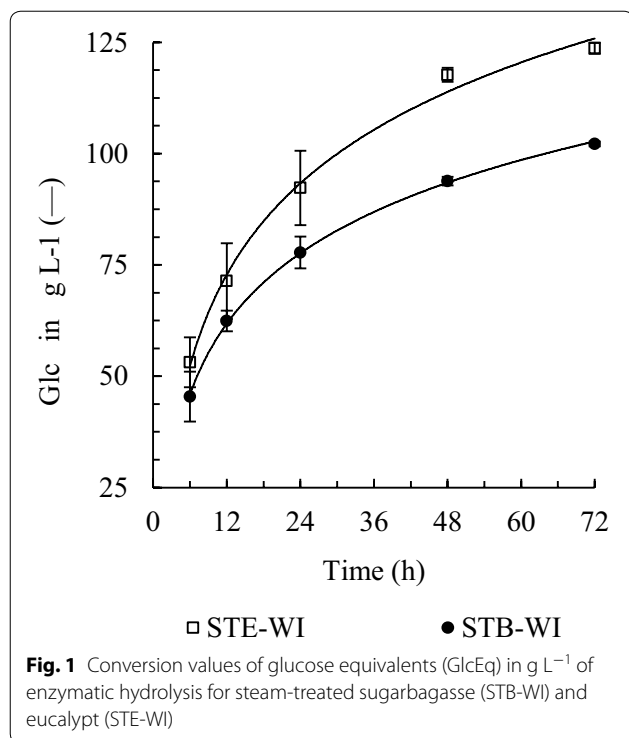
[a] Dehydration by-products from hexoses, quantified as hydroxymethylfurfural

[b] Dehydration by-products from pentoses, quantified as furfural

[c] Total anhydroglucose content plus its corresponding value in HMF

[d] Summation of anhydroxylose, anhydroarabinose, acetyl groups and the pentose equivalents that were detected as furfural

[e] Acid soluble lignin plus acid insoluble lignin

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Fig. 1 Conversion values of glucose equivalents (GlcEq) in g L^{-1} of enzymatic hydrolysis for steam-treated sugarbagasse (STB-WI) and eucalypt (STE-WI)

Fig. 2 Reactor vessel in which the enzymatic hydrolysis of steam-treated substrates were carried out at 20 wt% TS using Cellic CTec3

These hydrolysis procedures were carried out in a Labfors 5HT BioEtOH reactor with a total capacity of 3.2 L using shaft with multiple stirrers to ensure proper mixing of the substrate slurry (Fig. 2).

Ramos et al. [2] produced steam-exploded substrates from sugarcane bagasse at 180 °C for 10 min in the presence of 9.5 mg phosphoric acid g^{-1} (dry basis). Enzymatic hydrolysis was performed in shake flasks at 20 wt% TS with an EL of 62.5 mg of Cellic CTec2 g^{-1} cellulose. Despite the use of an exogenous acid catalyst at different pretreatment conditions, 76.8 g L^{-1} glucose was produced in 72 h of hydrolysis while 103 g L^{-1} was achieved with STB-WI in this study using the Labfors 5HT BioEtOH bioreactor under otherwise identical reaction conditions. This better hydrolysis performance can be explained by differences in substrate accessibility but also by differences in the hydrolysis procedure, since the later glucose release was obtained under stepwise substrate addition (fed-batch feeding) and a more efficient mechanical agitation.
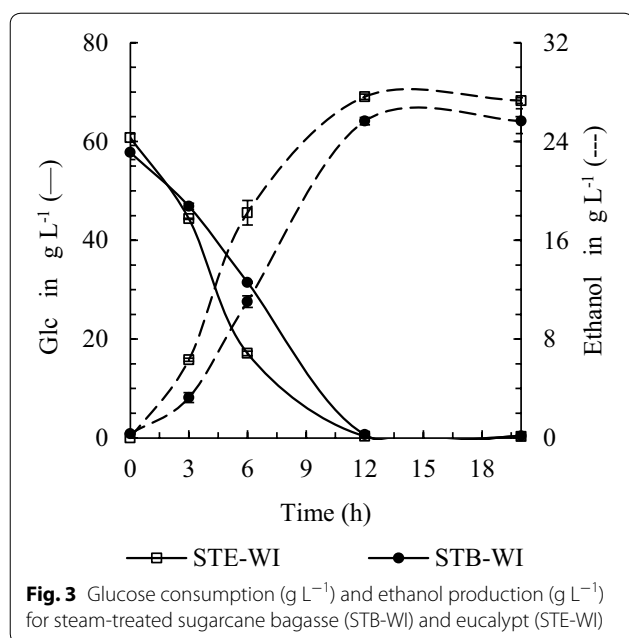
Martins et al. [30] produced cellulosic ethanol from sugarcane bagasse after pretreatment by alkaline hydrogen peroxide. Enzymatic hydrolysis for 24 h using 15 FPU g^{-1} (*Trichoderma reesei* ATCC 26921) plus 25 CBU g^{-1} (*Aspergillus niger*) produced 55.2, 69.6 and 78.5 g L^{-1} glucose at 10, 15 and 20 wt% TS, respectively. In another study, Horn and Eijsink [31] pretreated hardwood poplar by steam explosion at 210 °C for 10 min (log R$_O$ of 4.24)

and the resulting substrate was hydrolysed for 24 h with 25 FPU g^{-1} TS of Econase, resulting in 33, 34 and 36 g L^{-1} glucose at 10, 12.5 and 15 wt% TS, respectively. In this work, STB-WI reached similar values of 75.27 g L^{-1} glucose after 24 h of hydrolysis while STE-WI resulted in an even better glucose yields of 98.18 g L^{-1}. However, in both cases, hydrolysis was carried out at 20 wt% using only 13.3 FPU g^{-1} TS of Cellic CTec3.

Fermentation

The fermentation trials were performed with slightly different initial glucose concentrations of 58 and 60 g L^{-1} for STB-WI and STE-WI, respectively (Fig. 3). At the end of this process, 25.65 g L^{-1} ethanol and 87.1 % fermentation efficiency were obtained from STB-WI while STE-WI yielded 27.31 g L^{-1} ethanol for an efficiency of 88.1 %. Glucose consumption was complete after 12 h, suggesting that this process could have been interrupted at this time without any loss in fermentation efficiency. The corresponding ethanol productivities were 1.3 g L^{-1} h^{-1} for STB-WI and 1.4 g L^{-1} h^{-1} for STE-WI after 24 h of total fermentation time. However, in 12 h of fermentation,

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Fig. 3 Glucose consumption (g L^{-1}) and ethanol production (g L^{-1}) for steam-treated sugarcane bagasse (STB-WI) and eucalypt (STE-WI)

these values increased to 2.1 and 2.3 g L^{-1} h^{-1}, respectively. Martins et al. [30] fermented substrate hydrolysates containing 60 g L^{-1} glucose using an industrial strain of *S. cerevisiae* and obtained 1.6 g L^{-1} h^{-1} ethanol in 16 h for a fermentation efficiency of 84.5 %. However, this study involved pretreated substrates that were derived from sugarcane bagasse by alkaline hydrogen peroxide pretreatment.

Romaní et al. [32] produced cellulosic ethanol from pretreated substrates that were produced by non-isothermal autohydrolysis of *E. globulus* chips. In this pretreatment procedure, the reactor vessel was heated up to 210 °C and, when this temperature was reached, the heating system was disabled and the reactor was cooled down to ambient temperature for a total pretreatment time of 50 min. This way, pretreated substrates were produced and whole pretreatment slurries were submitted to SSF for cellulosic ethanol production. After 120 h of SSF at 15.6 wt% TS using 22.5 FPU g^{-1} of Cellic CTec2, 500 UI g^{-1} of Cellic HTec2 and 1.8 g L^{-1} *S. cerevisiae* PE-2, the resulting ethanol productivity after 72 h was 0.63 g L^{-1} h^{-1} for a fermentation efficiency of 94.7 %. Also, when a 24 h pre-hydrolysis step was used prior to simultaneous saccharification and fermentation (pSSF), the corresponding ethanol productivity and fermentation efficiency were lower at 0.55 g L^{-1} h^{-1} and 92.1 %, respectively. Since pSSF usually gives better ethanol productivities than SSF [23, 33], this unexpected result was probably due to the presence of inhibitors in the fermentation media. By contrast, Neves et al. [24] used water-washed steam-exploded sugarcane bagasse (195 °C for

7.5 min) for cellulosic ethanol production under both SSF and pSSF configurations and the latter reached the same ethanol concentration in half of the time required by the former, which corresponded to ethanol productivities of 0.39 and 0.63 g L^{-1} h^{-1}, respectively. However, these results were obtained in the absence of fermentation inhibitors using low enzyme loadings of Cellic CTec2 and the *S. cerevisiae* strain Thermossac Dry for fermentation. In the SHF configuration used in the present study, the total conversion time for both bagasse and eucalypt substrates was 84 h (72 h for hydrolysis and 12 h for fermentation). Therefore, the corresponding ethanol productivities were much lower at 0.31 and 0.33 g L^{-1} h^{-1}, respectively. However, these values cannot be compared to those described above because the strategy used for fermentation was completely different.

Comparison between substrates

When the results obtained in this work were projected to 1 t (1000 kg) of processed biomass, it seemed that *E. urograndis* was the best biomass for cellulosic ethanol production (Table 2). These projections were calculated by considering the recovery yield of water-washed steam-treated fractions (STB-WI and STE-WI), the amount of glucose that was obtained after enzymatic hydrolysis and the corresponding fermentation efficiency for ethanol production. Eucalypt chips presented a slightly lower recovery yield of the STB-WI fraction but its susceptibility to enzymatic hydrolysis was considerably higher, yielding 17 % more glucose than the corresponding STB-WI fraction after 72 h of hydrolysis. On the basis of these, 218.5 L t^{-1} ethanol were obtained from *E. urograndis* chips while bagasse reached only 178.0 L t^{-1} ethanol. These numbers are considerably high if compared to the theoretical amount of ethanol that can be produced from both sugarcane bagasse and eucalypt steam-exploded substrates, whose maximum fermentation efficiency produces 204.4 and 248.0 L t^{-1} ethanol, respectively. Nevertheless, it is important to mention that 7.1 t of sugarcane are necessary to produce 1 t of dry sugarcane bagasse [5, 6].

According to Ramburan [34], sugarcane reaches harvest maturity after 12 months in equatorial and hot tropical regions. By contrast, the complete eucalypt harvest cycle usually takes seven years [35]. One effective way to achieve a more realistic comparison between these two biomass types is to consider their average annual productivities, which are projected to be around 20.16 t ha^{-1} year^{-1} for sugarcane bagasse [5] and 25.92 t ha^{-1} year^{-1} for *E. urograndis* on wet basis [36]. This later average annual productivity, which was calculated using a density of 576 kg m^{-3}, is very similar to the average value reported Dougherty and Wright [37] for *Eucalyptus* spp..

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Table 2 Cellulosic ethanol production by ton of processed biomass

Data	Sugarcane bagasse	*E. urograndis*
Steam explosion (% STE-WI)[a]	64.0	61.4
Glucose release after hydrolysis (g L^{-1} Glc)[b]	103	125
Total amount of glucose release (g kg^{-1} Glc)	515	625
Fermentation efficiency (%)[c]	87.1	88.1
Ethanol production per ton (L t^{-1})	178.0	218.5
Theoretical production per ton (L t^{-1})	204.4	248.0
Biomass productivity (t ha^{-1} year^{-1})[d]	20.16	25.92
Ethanol productivity from 1st generation	5760.0 L ha^{-1} year^{-1}	–
Ethanol productivity from 2nd generation (L ha^{-1} year^{-1})	1794.5	2832.2
Total ethanol productivity (L ha^{-1} year^{-1})	7554.5	2832.2

[a] Sugarcane bagasse and eucalypt were pretreated at 195 °C for 7.5 min and at 210 °C for 5 min, respectively

[b] Hydrolysis of steam-treated substrates in a Labfors 5HT reactor using 20 wt% TS and 62.5 mg of Cellic CTec3 g^{-1} TS at 50 °C for 72 h (2.5 L total volume)

[c] 1 g L^{-1} of *S. cerevisiae* Thermosacc Dry at 37 °C for 12 h

[d] Data available from Comércio Ambiental [36] and Carvalho et al. [5] for *E. urograndis* chips and sugarcane bagasse, respectively

Hence, eucalypt plantations are able to achieve cellulosic ethanol productivities of 2832.2 L ha^{-1} year^{-1} while sugarcane bagasse would produce a 36.6 % lower value of around 1794.5 L ha^{-1} year^{-1}. However, sugarcane has the advantage of producing ethanol from first generation, which is estimated to be 80 L t^{-1} in average [38]. Based on the average biomass productivity of 72 t ha^{-1} year^{-1} that was obtained in the last Brazilian harvest season [5], this corresponds to 5760.0 L ha^{-1} year^{-1} of ethanol on wet basis or a total of 7554.5 L ha^{-1} year^{-1} if both first and second generation technologies are added together. Hence, under the conditions used in this study, bagasse glucans showed the potential to boost the ethanol production from sugarcane culms by 31 %, from the 80 L t^{-1} of first generation to a total production of 105 L t^{-1}, this without considering the yield increments that are expected to arise from the C5 stream as well as from the utilization of other harvest residues for the same purpose, such as sugarcane leaves and tops. These two possibilities also apply to eucalypt wood chips, whose pruning, selective cut and post-harvest residues are estimated to reach 30 t ha^{-1} year^{-1} [39], this without including the roots because its collection is both economically and environmentally unfeasible [40].

Gonzalez et al. [41] performed an economic analysis of cellulosic ethanol production from eucalypt biomass and the profitability of the process was most sensitive to the biomass cost, followed by the biomass carbohydrate content and the enzyme cost. Regarding the biomass cost, the Brazilian average market price for industrial eucalypt chips in 2015 was R$ 77.00 t^{-1}, whereas sugarcane bagasse was selling for roughly R$ 150 t^{-1} [42, 43]. In fact, both prices varied a lot throughout the year but sugarcane bagasse was always more expensive due to its increased demand for bioenergy applications such as cogeneration. Hence, both biomass types showed good potential for cellulosic ethanol production but *E. urograndis* chips resulted in the highest yields of 218.5 L ethanol t^{-1}, with the added value of being found at a relatively low cost.

Conclusion

Pretreatment of sugarcane bagasse and eucalypt chips by steam explosion resulted in high yields of enzymatic hydrolysis at high total solids and a relatively low enzyme loading. However, the glucose yield was higher for steam-treated eucalypt, giving yields 25 % higher than those of steam-treated bagasse. With regard to fermentation, both hydrolysates were readily fermented in good yields by an industrial yeast strain of *S. cerevisiae*, achieving productivity values higher than 2 g L^{-1} h^{-1} after only 12 h. However, when these results were projected to 1 t of processed biomass, eucalypt chips showed the greatest potential for bioconversion, giving around 40 L of ethanol t^{-1} more than sugarcane bagasse.

Experimental

Sugarcane bagasse was obtained from the Cane Technology Center (CTC, Piracicaba, SP, Brazil) and industrial chips of *E. urograndis* were provided by EMBRAPA-*Florestas* (Colombo, PR, Brazil). The commercial enzyme preparation used for hydrolysis (Cellic® CTec3) was provided by Novozymes Latin America (Araucária, PR, Brazil). The yeast used for fermentation was Thermosacc® Dry, a commercial *Saccharomyces cerevisiae* preparation produced by Lallemand Specialties Inc. (Milwaukee, WI, EUA).

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Chemical analysis of lignocellulosic materials

The materials were subjected to extraction in a Soxhlet apparatus for the determination of their total extractive contents following the NREL/TP-510-42619 method [44]. This way, milled eucalypt chips (40–60 mesh) were extracted with ethanol 95 % while milled bagasse (40–60 mesh) was extracted with water followed by ethanol 95 %. The moisture and ash contents were determined according to the NREL/TP-510-42621 [45] and NREL/TP-510-42622 [46] methods, respectively. The chemical composition of both cellulosic materials was determined according to the NREL/TP-510-42617 [47] and NREL/TP-510-42618 [48] methods, before and after pretreatment, generating the acid insoluble lignin (determined gravimetrically) and the acid soluble lignin (determined by UV spectroscopy), respectively. Carbohydrate composition was determined in the resulting acid hydrolysates by HPLC using a Shimadzu (Kyoto, Japan) HPLC model LC20AD and an Agilent (Santa Clara, CA, EUA) Hi-Plex-H column at 65 °C that was eluted with 5 mmol L^{-1} H_2SO_4 at a flow rate of 0.6 mL min^{-1}. Detection was carried out by both refractive index (Shimadzu, model RID10A) and UV spectrophotometry using a photodiode array detector (Shimadzu, model SPD-M10Avp). The quantification was performed by external calibration (0.01–1 g L^{-1}) using a series of standard solutions for each component of interest: cellobiose, glucose, xylose, arabinose, formic acid, acetic acid, furfural and hydroxymethylfurfural, the latter two used for controlling the dehydration of pentoses and hexoses, respectively.

Pretreatment by steam explosion

Pretreatment by steam explosion was carried out in a 10 L stainless steel reactor that was provided with sensors to control reaction parameters such as the pretreatment pressure, temperature and time. The steam explosion experiments were performed under pre-optimized conditions to recover most of the three main biomass components and to increase the susceptibility of the resulting substrate to enzymatic hydrolysis and fermentation. These conditions correspond to autohydrolysis at 195 °C for 7.5 min for sugarcane bagasse [49] and 210 °C for 5 min for eucalypt chips (unpublished data). The severity factor (log R_O) of these pretreatment conditions was calculated according to Overend e Chornet [50], where T (°C) is temperature and t (min) is residence time in the reactor (Eq. 1).

$$\log R_O = e^{\left(\frac{T-100}{14.75}\right)} . t \tag{1}$$

After pretreatment, the resulting steam-treated material was immediately filtered through a cheese cloth using a Büchner funnel. The fiber retentate was suspended in water at 5 wt% TS, washed for 1 h at room temperature under mechanical stirring and filtered once again. The resulting steam-treated materials were named water-insoluble steam-treated sugarcane bagasse (STB-WI) and water-insoluble steam-treated eucalypt (STE-WI). Pretreatment mass yields were calculated by difference before and after pretreatment, always in relation to the dry mass of the corresponding materials.

Enzymatic hydrolysis

Enzymatic hydrolyses were carried out in a Labfors 5 BioEtOH reactor (Infors-HT, Bottmingen, Switzerland) using shaft with multiple stirrers during 72 h at 50 °C and 150 rpm. Cellic CTec3 was used at an EL of 62.5 mg g^{-1} dry substrate in 50 mmol L^{-1} acetate buffer, pH 5.2. EL was expressed in relation to the wet mass of the enzyme preparation and its total cellulase activity was determined according to Ghose [51]. The reactor was fed with pretreated material by adding 5 wt% TS at every 1.5 h, reaching 20 wt% TS in 4.5 h for a total reaction volume of 2.5 L. Aliquots of 0.5 mL were withdrawn in different times and these were analysed by HPLC as mentioned earlier. The results were expressed in glucose equivalents (GlcEq = glucose + cellobiose × 1.0526) using both glucose and cellobiose solutions as reference standards (0.25 to 5 g L^{-1}). Hydrolysis yields were expressed in relation to the glucan content of the pretreated substrates.

Fermentation

Fermentation was performed in duplicate at 35 °C for 20 h by applying a *S. cerevisiae* Thermosacc Dry yeast strain to the substrate hydrolysates obtained after enzymatic hydrolysis. The fermentation medium contained 20 mL of hydrolysate, 50 mmol L^{-1} acetate buffer pH 4.8, 1.0 g L^{-1} yeast extract, 0.5 g L^{-1} $(NH_4)_2PO_4$, 0.025 g L^{-1} $MgSO_4·7H_2O$ and 1.0 g L^{-1} of Thermosacc Dry for a total reaction volume of 100 mL. Aliquots of 0.3 mL were withdrawn in different times and these were analysed by HPLC as mentioned earlier, with glucose and ethanol solutions as reference standards (0.25–5 g L^{-1}). Production of ethanol was expressed in g L^{-1} while the corresponding ethanol productivity (or the rate of ethanol production) was calculated in g L^{-1} h^{-1}.

Abbreviations

EL: enzyme loading; FPU: filter paper unit; GlcEq: glucose equivalents (glucose + cellobiose × 1.0526); HPLC: high performance liquid chromatography; log R_O: severity factor; NREL: National Renewable Energy Laboratory; PDA: photodiode array; pSSF: pre-hydrolysis followed by simultaneous saccharification and fermentation; RID: refractive index detector; SHF: separated hydrolysis and fermentation; SSF: simultaneous saccharification and fermentation; STB-WI: water-insoluble, steam-treated sugarcane bagasse; STE-WI: water-insoluble, steam-treated eucalypt; TS: total solids.

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