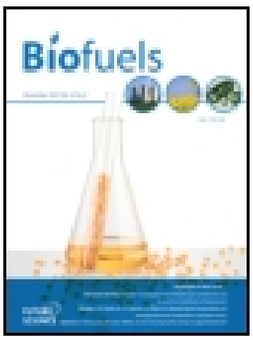

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Fungal pretreatments improve the efficiency of saccharification of *Panicum prionitis* Ness biomass

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ABSTRACT

Second-generation bioethanol derived from native perennial grasses offers a promising alternative for biofuel, especially when the biomass avoids land-use competition for crop production. *Panicum prionitis* Ness is a native perennial C4 grass predominant in soils of the Delta del Paraná River, Argentina. Its forage quality (palatability and digestibility) for livestock is low because of its substantial lignin content. In this work, we evaluated different pretreatments (phosphoric acid, ligninolytic enzymes and fungal secretomes) aimed to degrade lignin and improve cellulose hydrolysis efficiency. Results show that 2-day pretreatments with fungal secretomes highly improve release of fermentable sugars compared with conventional pretreatments. Although *Pycnoporus sanguineus* displayed a greater contribution than *Ganoderma applanatum* to the pretreatment, the latter triggered the highest final yield, achieving a hydrolysis of 47.5% of cellulose when added to green tissue. These results strengthen the feasibility of using *Panicum prionitis* biomass in a low-polluting bioethanol production process.

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Biomass; ligninolytic pretreatments; lignocellulosic materials; rangelands; white rot fungi; *Pycnoporus sanguineus*; *Ganoderma applanatum*

Introduction

Biofuels based on plant biomass are one of the best candidates to supply the increasing global energy demand. Plants use solar power to convert carbon dioxide and water into sugars, which can be used in fermentation reactions to produce high-energy molecules. While first-generation biofuels derive from raw material that competes with the food industry (corn, sugar beet, sugarcane and sorghum for bioethanol production, and oil crops – soybean and rapeseed – for biodiesel production), second-generation biofuels are produced using lignocellulosic materials (energy crops, rangeland plants, crop residues). These exhibit many advantages such as lower resource requirements, higher energy potential, no competition with the food industry, non-agricultural land use, and eroded land recovery [1–4]. Biomass with low lignin and high cellulose and hemicellulose contents is the most valuable for this type of biofuel [5].

Particularly, the use of perennial grassland for second-generation biofuels shows multiple benefits, among them high photosynthetic and growth rates, low environmental impact with a negative carbon balance [6,7], and no or little fertilization requirement due to the high nitrogen use efficiency of C4 plants [8–10]. However, because of the structurally complex nature of lignin, industrial bioconversion into bioethanol is an interesting challenge [4].

Several lignocellulosic species have been studied as potential biofuel materials, *Miscanthus* spp. and *Panicum virgatum* being the two most important energy crops worldwide [11]. Growing native perennial species on marginal lands not currently farmed represents a good option for climate mitigation [12]. *Spartina argentinensis*, which grows in rangelands and does not need to be sown, has also been reported as a competent bioethanol resource [13].

Another C4 grass from a rangeland is *Panicum prionitis* Ness, a dense, sturdy, summer-growing, perennial and rhizomatous plant, characteristic of the Chaco-Pampean plain of Argentina. *Panicum prionitis* is the dominant species in floodable soils near great watercourses, presenting a biomass production that varies between 10 and 18 t ha^{-1} $year^{-1}$ [14]. Due to the characteristics of the land, these communities present low population density, with scarce or almost null economic activity, where the rearing and wintering of cattle is the main activity developed. However, the low palatability and digestibility of *P. prionitis* leaves, as a consequence of the accumulation of lignin at advanced stages, make fire the most economical and widespread management method to take advantage of the tender shoots [15]. From an ecological and environmental point of view, the use of fire is not a recommendable practice, since it releases CO_2 into the atmosphere. Burning should be avoided to mitigate

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the increase in the concentration of greenhouse gases, and one alternative practice could be the cutting and subsequent use of the biomass for the production of second-generation bioethanol [16,17].

Pretreatment is a fundamental step in the production of bioethanol from lignocellulosic biomass, and constitutes the bottleneck of the global process. Effective conversion of the carbohydrate polymers to simple sugars relies on numerous factors including composition and structure of the feedstock, pretreatment used, type and loading of enzyme, cellulose crystallinity, and available surface area. In their native state, cellulose and hemicellulose are largely protected from enzymatic degradation. Their inaccessibility to hydrolyzing enzymes is mainly due to the associations of these polymers with lignin and with each other, which act as a barrier. Pretreatment is necessary to enhance hydrolytic enzyme action that will produce simple sugars from lignocellulosic biomass. The major criteria for efficient pretreatment consist of lignin and hemicellulose removal, increase in accessible surface area and porosity, and decrease in cellulose crystallinity and polymerization degree with the lowest concentrations of inhibitors that inhibit biocatalytic reactions [4]. In this way, the cellulose is exposed to the action of hydrolytic enzymes, thereby increasing the efficiency of the subsequent saccharification step. Thus, the pretreatment may be essential to effectively prepare the cellulose of lignocellulosic biomass for high-yield enzymatic hydrolysis [18].

Biomass pretreatment can be carried out by different methods such as chemical (acid, alkali, ionic liquid, ozonolysis), physical (microwave, grinding and milling), physicochemical (steam explosion, wet oxidation, liquid hot water) and biological (ligninolytic enzymes or microorganisms) pretreatments. Usually, a combination of these processes is used to increase the efficiency [19].

Pretreatment with fungal microorganisms is shown to be a promising technique due to several advantages over physical/chemical pretreatments, such as reaction and substrate specificity, low energy requirements, and no production of toxic compounds, resulting in the biomass becoming more environmentally friendly [19–21]. It is, however, limited by the reaction time and requires careful monitoring of fungal growth conditions, as well as huge facilities for large-scale development [18].

White rot fungi have developed the ability to completely mineralize lignin by a degradation mechanism that involves an enzymatically regulated oxidative process [22]. Many of these enzymes are secreted to the extracellular media because these fungi degrade cellulose and hemicellulose, and utilize their products as sources of carbon and energy [23]. The secreted proteins (the 'secretome') include hydrolytic, non-hydrolytic and oxidative-ligninolytic enzymatic systems [24].

In the present study, we evaluated *Panicum prionitis* Ness as a lignocellulosic biomass for bioethanol production. We assayed two types of biomass (green and senescent leaves), and different pretreatments to degrade lignin as a previous step for saccharification: (i) phosphoric acid (chemical pretreatment), (ii) commercial ligninolytic enzymes (laccase, manganese peroxidase and lignin peroxidase), and (iii) secretomes from two white rot fungi (*Pycnoporus sanguineus* and *Ganoderma applanatum*). Our results suggest that *P. prionitis* biomass may be a suitable substrate for second-generation bioethanol production employing a short and non-contaminating biological pretreatment.

Materials and methods

Biomass, chemicals and enzymes

Panicum prionitis Ness plants were originally collected from their habitat at Estancia 'La Catalina' (32°52'43.04"S, 60°35'0.33"W; Victoria, Entre Ríos, Argentina). They were transplanted to a plot at the Experimental Field in the Facultad de Ciencias Agrarias of the Universidad Nacional de Rosario (Zavalla, Santa Fe). Leaves were harvested, oven dried (60 °C) to constant weight, ground to 7 × 3 × 1 mm pieces (gross grind) or 3 × 2 × 1 mm pieces (fine grind), and stored in plastic bags at room temperature. Senescent leaves can remain attached to the plant for long periods and can constitute a significant amount of biomass (data not shown), so they were collected and treated separately.

Chemicals and enzymes used were purchased from Sigma-Aldrich Argentina unless stated otherwise. Enzymes employed in this study were: β-glucosidase from almonds (49,290), hemicellulose from *Aspergillus niger* (H2125), cellulase from *A. niger* (C1184), lignin peroxidase (42,603) (LiP), manganese peroxidase (93,014) (MnP), and *Trametes versicolor* laccase (51,639).

Chemical composition of biomass

Chemical composition (percentage of cellulose, hemicellulose and lignin) of green and senescent biomass from *P. prionitis* was determined by the detergent system used to evaluate the nutritional value of foods for ruminants [25]. Differences among types of biomass were assessed comparing two groups by Student's *t*-test.

Fungal species for biomass pretreatments

Two white rot fungi were assessed: *Pycnoporus sanguineus* (Fr.) Murr. and *Ganoderma applanatum* (Pers) Pat. The fungal basidiocarps were collected from different trees at the Experimental Campus, Zavalla. Fungi were isolated by extracting mycelia from basidiocarps in a

laminar flow chamber and inoculated in Petri dishes with potato dextrose agar (PDA) solid medium. Plates were incubated for 7 days, in darkness, in an oven at 28 °C. Inocula were kept at 4 °C and subcultured periodically.

Biomass pretreatments and saccharification

All pretreatments were performed over three biological replicates of *Panicum prionitis* biomass and were followed by a saccharification step. Glucose release was measured after the whole process. Blanks correspond to the experimental units that account for endogenous sugar concentration of all the components of each reaction mixture. Controls correspond to the experimental units that were only subjected to the saccharification step, in order to be able to quantify the contribution of each pretreatment (by the subtraction of controls from total released glucose) to the global process. Preparation of blanks and controls is described in the following sections.

Chemical biomass pretreatment

Fifteen milligrams of ground dried green and senescent leaves of *P. prionitis* were placed in Eppendorf tubes containing 500 μL of 85% v/v H_3PO_4 and processed as described in Larran et al. 2015 [13]. Blanks and controls contained distilled water instead of H_3PO_4.

Biological biomass pretreatment with commercial enzymes

Fifteen milligrams of ground dried green or senescent leaves of *P. prionitis* were placed in Eppendorf tubes, and 0.0025 U of a single ligninolytic enzyme (LiP or MnP or laccase) diluted in 250 μL of 50 mM sodium acetate buffer pH 4.0 was added. Blanks and controls contained the buffer solution without the enzymes. Tubes were incubated at 37 °C for 48 h.

Biological biomass pretreatment with fungal secretomes

A disk of 1 mm^3 from the peripheral region of fungus actively growing on PDA plates was inoculated into 20 mL of potato dextrose (PD) liquid medium and incubated at 28 °C with rotary shaking (120 rpm) for 7 days. The supernatant of each fungus was filtered under sterile conditions. Erlenmeyer flasks containing 50 mg of ground dried green or senescent leaves of *P. prionitis* were incubated at 37 °C for 48 h, after the addition of 0.5 mL of fungus supernatant diluted in 4.5 mL of 50 mM sodium acetate buffer pH4. Blanks and controls were prepared adding a boiled fraction of the fungus supernatant (denatured enzymes).

Biomass saccharification

The saccharification step was the same for all pretreatments, in order to accurately compare the efficiencies of the latter. Green or senescent pretreated leaves were incubated with a mixture of cellulase, hemicellulase and ß-glucosidase, previously filtered to remove stabilizing glucose [see 13]. Enzymes (0.4 U each) were dissolved in 500 μL of 100 mM sodium acetate buffer pH 6.0 immediately prior to use. Blanks were prepared with a boiled enzyme mixture. After a 48-h incubation at 50 °C, a 20-μL aliquot was taken for glucose determination using an enzymatic glycemia kit (Wiener Lab, Rosario, Argentina).

Values of released glucose of blanks were subtracted from treated experimental units in order to obtain the total glucose released in the process. Simultaneously, values of released glucose of controls were subtracted from treated experimental units in order to calculate how much the pretreatments contributed to the total glucose release. Results are expressed as milligrams of total glucose released per gram of *P. prionitis*. The percentage of hydrolyzed cellulose was calculated as described in Larran et al. [13].

Statistical analysis

Data from the experiments of pretreatments were tested using two-way analysis of variance (ANOVA). Minimum significant differences were calculated by the Holm–Sidak Test ($\alpha = 0.05$) using the Sigma Stat Package.

Results and discussion

Chemical composition of Panicum prionitis *biomass*

Panicum prionitis biomass remains an interesting material for bioethanol production, according to Van Soest assay. Polysaccharides account for almost 70% of the dry matter contents (cellulose: 33–35%; hemicellulose: 32–35%), and lignin contents are close to 7% (Table 1). The content of cellulose and lignin is higher in senescent leaves, while the hemicellulose content is higher in green leaves. This phenomenon was observed in previous studies with *Spartina argentinensis* biomass [13], showing a significant change of cell wall composition at advanced stages. Lignin content may vary because of multiple factors such as cell tissue, cell stage, environmental conditions and plant age [26]. According to chemical composition of *P. prionitis* leaves, Feldman 2016 [27] estimated that 9830 L ha^{-1}

Table 1. Chemical composition of *Panicum prionitis* Ness biomass, expressed as percentages of dry weight. Statistically significant differences were found between the two types of biomass (Student's *t*-test, $p < 0.05$).

Biomass	Cellulose	Hemicellulose	Lignin
Green leaves	33.06 ± 0.06	35.45 ± 0.22	6.28 ± 0.07
Senescent leaves	35.46 ± 0.31	32.6 ± 0.23	7.4 ± 0.11

of bioethanol could be obtained, which represents a high yield considering the values reported for other lignocellulosic feedstocks [28].

Pretreatments

The basis of our study was to compare the effectiveness of three pretreatments – two biological pretreatments (fungal secretomes and commercial ligninolytic enzymes) and one chemical pretreatment (phosphoric acid) – of *P. prionitis* biomass, and its prospective utilization as a substrate for bioethanol production.

Pretreatments with phosphoric acid and commercial ligninolytic enzymes

The amount of released glucose using phosphoric acid was 5.0 ± 0.3 and 0.4 ± 0.2 mg per g of green leaves and senescent leaves, respectively (Figure 1). These values are quite low, especially considering that the acid pretreatments are reported as one of the most efficient among conventional methods [29].

In the case of using ligninolytic enzymes, we assayed three enzymes separately: laccase, MnP and LiP. The enzymatic pretreatment increases the released glucose by between 13 and 37% in comparison with the control samples treated only with the saccharification cocktail. This result suggests that polysaccharide exposure is increased by the ligninolytic enzyme pretreatment, giving better access to the substrates in the next step. Unlike Larran et al. [13], we did not find statistically significant differences using these pretreatments on green and senescent biomass, despite the similar cell wall composition of each sample in the two species. These results support the hypothesis that not only the contents of lignin, hemicellulose and cellulose, but also the differential cross-linking between them, may influence the efficiency of the pretreatment and the subsequent biomass saccharification.

Pretreatments with fungal secretomes

The amount of sugar released from *P. prionitis* biomass using fungi secretomes as agents for pretreatments was notably higher than that observed with the chemical (phosphoric acid) and biochemical (ligninolytic enzymes) pretreatments. The highest efficiency was found with the *G. applanatum* secretome, which was up to 15-fold higher than conventional chemical pretreatment. *Ganoderma applanatum* secretome reached about 70 mg of released glucose per g of *P. prionitis* biomass, while the results with *P. sanguineus* supernatant were half and one tenth of this value for senescent and green biomass, respectively (Figure 1).

The pretreatment effect on glucose release varies according to the fungus species and the composition of biomass. The pretreatment with the highest contribution was reached by using the secretome of *P. sanguineus* on both types of *P. prionitis* biomass (34% for green leaves, and 59% for senescent leaves) (Figure 2). However, the highest values of released glucose for the complete process (pretreatment plus saccharification) were observed when the secretome from *G. applanatum* was assayed (74.7 and 66.7 mg of released glucose per gram of *P. prionitis,* for green and senescent leaves, respectively).

It is known that white rot fungi attack lignocellulosic materials, simultaneously degrading cellulose, hemicellulose and lignin [30]. The differences observed in this work may be due to the secretion of a specific enzyme consortium by each fungus, which might contribute differentially to the pretreatment and saccharification steps. In other words, we can assume that the enzymes secreted by *P. sanguineus* would contribute in a more important way to the pretreatment, while the enzymes secreted by *G. applanatum* would show a higher activity in the saccharification. In fact, when avoiding the use of commercial enzymes in the saccharification step, released glucose values were higher for *G.*

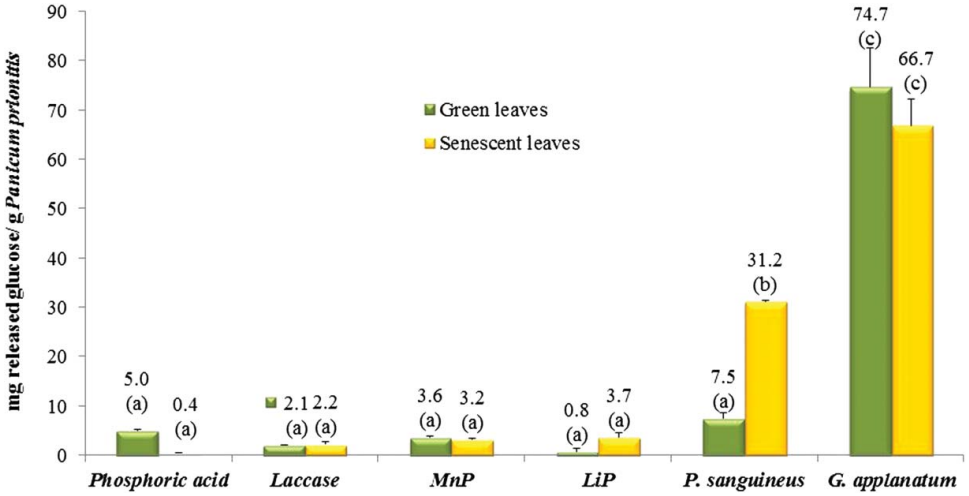

Figure 1. Glucose released after pretreatment and saccharification process on green and senescent leaves of *Panicum prionitis*. The error bars represent the standard deviation. Different letters indicate statistically significant differences.

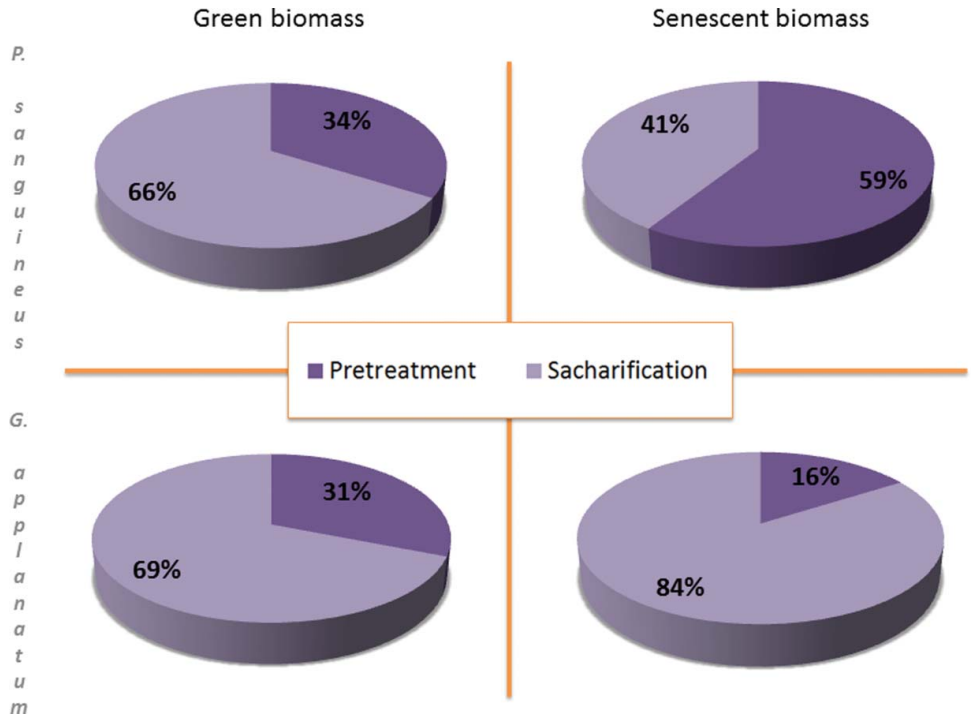

Figure 2. Contribution of pretreatment and saccharification to total glucose release. Percentages for *Pycnoporus sanguineus* and *Ganoderma applanatum* are presented in the top and bottom graphics, respectively. The pretreatment with *P. sanguineus* secretome is most effective on senescent leaves, while it is half effective on senescent leaves compared to green leaves with *G. applanatum* secretome.

applanatum than for *P. sanguineus*. However, in both cases the global efficiency of glucose release was significantly lower than when using cellulolytic commercial enzymes (data not shown).

Considering that *G. applanatum* grows naturally on living plant tissues, it seems reasonable that the percentage of its contribution to the pretreatment degrading the senescent biomass is substantially lower than on green leaves. Otherwise, with the secretome from *P. sanguineus*, which grows preferentially on dead trees [31,32], the highest contribution to the pretreatment was reached on senescent leaves, which resulted in a higher global efficiency, as observed in Larran et al. [13]. However, the action of the secretome of *P. sanguineus* on *S. argentinensis* biomass triggered higher glucose release values than those obtained in this study. This may be due to the differential interactions between polysaccharides (cellulose and hemicellulose) and lignin, as well as the presence of inhibitory substances whether endogenous to the biomass or produced during the pretreatment and/or saccharification process.

On the other hand, the grinding conditions also had an impact on the efficiency of released glucose. Thus, when *P. prionitis* biomass was finely ground to produce smaller pieces, and it was pretreated with *G. applanatum* secretome (the most efficient pretreatment as previously described), an increase of 148% for green leaves and 140% for senescent leaves of free glucose was observed (Figure 3). This can be explained by a higher surface area being exposed to the lignocellulosic enzymes. Moreover, it is worth highlighting that ethanol production could be carried out from green and senescent tissues at the same efficiency, proving the feasibility of this process from the material harvested.

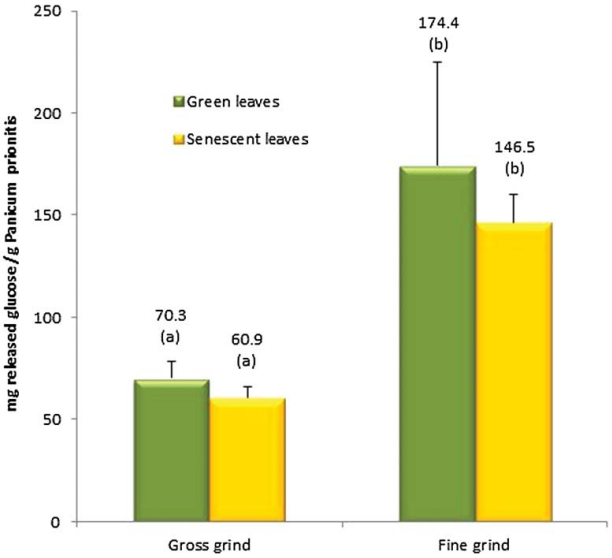

Figure 3. Comparison of the effect grinding size of biomass treated with *Ganoderma applanatum* secretome on glucose release. The error bars represent the standard deviation. Different letters indicate statistically significant differences.

As described in the Materials and methods, fungi used to evaluate the secretome pretreatment

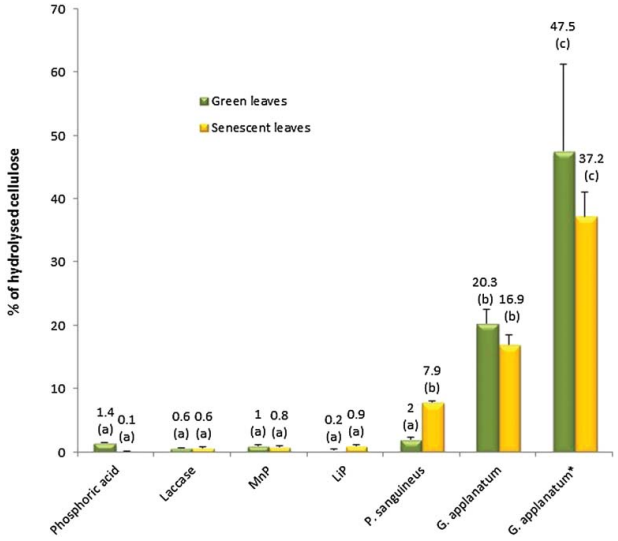

Figure 4. Percentage of hydrolyzed cellulose for all pretreatments. The error bars represent the uncertainty of the theoretical estimations (calculated by propagation). Different letters indicate statistically significant differences. The asterisk indicates finely ground biomass.

efficiency were grown in PD medium. It should be noted that, despite starting from a high glucose concentration in the secretome, the efficiency of obtaining fermentable sugars was higher than that obtained from conventional pretreatments. However, this basal glucose may cause an underestimation of the final efficiency of the process, since it has been reported that glucose can inhibit cellulase activity during the saccharification process [33]. Besides, lignocellulolytic enzymes secreted by white rot fungi are induced in the presence of lignocellulosic materials in the absence of glucose [34]. Thus, these results may be improved if the fungi could be grown in a medium containing *P. prionitis* leaves as the only source of carbon and energy.

Comparison of hydrolyzed cellulose by different pretreatments

Figure 4 shows a comparison of the percentages of hydrolyzed cellulose among all the pretreatments carried out in this study. As described above, biomass pretreated with secretomes from white rot fungi resulted in the highest values of released glucose, reaching a hydrolysis of almost 50% of the total cellulose when using the *G. applanatum* secretome on finely ground green leaves of *P. prionitis*. A similar hydrolysis percentage was obtained in our previous work for *P. sanguineus* secretome on senescent leaves of *S. argentinensis* [13]. Hence, the compatibility of each system secretome/biomass is critical to reach the best yields.

Conclusions

This work shows that *Panicum prionitis*, a grass growing on floodplain soils, may be considered a promising biomass source for second-generation bioethanol. Lignin degradation using fungal secretomes released almost 50% of glucose for fermentation. These results may contribute to generate new economic activities in these marginal areas. This is the first study about the potential use of *P. prionitis* biomass as a biofuel material, with the fungal secretome pretreatments being the most promising systems to evaluate on a larger scale. Further research is needed to optimize the saccharification process and apply this knowledge to generate environmentally friendly business.

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Disclosure statement

The authors declare that they do not have any conflict of interest.

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