Bioresource Technology 194 (2015) 320–325

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Evaluation of biological pretreatments to increase the efficiency of the saccharification process using *Spartina argentinensis* as a biomass resource

Alvaro Larran [a], Emiliano Jozami [a], Lionel Vicario [b], Susana R. Feldman [a,c], Florencio E. Podestá [b,d], Hugo R. Permingeat [a,*]

[a] Facultad de Ciencias Agrarias, Universidad Nacional de Rosario, Campo Experimental Villarino, S2125ZAA Zavalla, Santa Fe, Argentina
[b] Facultad de Ciencias Bioquimicas y Farmacéuticas, Universidad Nacional de Rosario, Suipacha 531, 2000 Rosario, Santa Fe, Argentina
[c] Consejo de Investigaciones Universidad Nacional de Rosario, Maipú 1165, 2000 Rosario, Santa Fe, Argentina
[d] Centro de Estudios Fotosintéticos y Bioquímicos (CEFOBI), Suipacha 570, 2000 Rosario, Santa Fe, Argentina

HIGHLIGHTS

- *Spartina argentinensis* could be a suitable C4 grass for bioethanol production.
- Fungal supernatants are better than commercial ligninolytic enzymes.
- *Pycnoporus sanguineus* triggered the highest amount of glucose release.

ARTICLE INFO

Article history:
Received 29 April 2015
Received in revised form 29 June 2015
Accepted 30 June 2015
Available online 4 July 2015

Keywords:
Biomass
Second generation bioethanol
Pretreatments
Lignocellulose
White rot fungi

ABSTRACT

Second generation bioethanol obtained from native perennial grasses offers a promising alternative for biofuel production, avoiding land use competition for crops production. *Spartina argentinensis* is a native perennial C4 grass with high photosynthetic rates, well adapted to halo-hydromorphic soils, though its forage quality (palatability and digestibility) for livestock is quite low due to its high lignin content. Hence, cattle raisers burn these grasslands frequently in order to stimulate the emergence of new leaves with higher digestibility for cattle feeding. Lignin is the main barrier to overcome in order to efficiently hydrolyze the cellulose for bioethanol production. In this work, we evaluate different pretreatments (phosphoric acid, ligninolytic enzymes and fungal supernatants) aimed to remove lignin and improving cellulose hydrolysis efficiency. Results show that pretreatment with *Pycnoporus sanguineus* supernatant improves fermentable carbohydrates availability, compared with a conventional chemical pretreatment, and that 56.84% of cellulose can be hydrolyzed using this pretreatment.

1. Introduction

Global energy demand is growing steadily, hence contributing to rising the concentration of greenhouse gases in the atmosphere as a result of increased combustion of fossil fuels. This fact has caused a renewed interest in alternative energy sources, within which biofuels intended for transportation are playing an important role considering that a large percentage of fuel demand are associated with transport (IEO, 2010).

Biofuel production has increased significantly over the last decades (Langeveld et al., 2014). The main contributors to such increase are bioethanol from sugar cane and starch crops (corn, sugar beets, sorghum, etc.) and biodiesel from oil crops (mainly soybean and rapeseed biodiesel). Using these crops for fuel production has aroused great controversy as such scenario raises the demand of these commodities (intended for human consumption), therefore increasing its prices. Moreover, crops derivation to biofuel seems to be far from a solution considering that if 100% of the world production of the main cereals and starch crops were used for bioethanol production (not a viable scenario) only 57–85% of the energy used for gasoline vehicles would be accounted (Rajagopal and Zilberman, 2007; FAO, 2008). Furthermore, many environmental assessments of these bioenergy sources have concluded that they would present disadvantages in terms of energy and carbon balance (Solomon et al., 2007; FAO, 2008). Therefore,

* Corresponding author. Tel.: +54 341 4970080.
E-mail address: hperming@unr.edu.ar (H.R. Permingeat).

http://dx.doi.org/10.1016/j.biortech.2015.06.150
0960-8524/

there is a growing demand for new alternatives of bioethanol from non-food biomass (second generation bioethanol). Some of these alternatives are energy crops, high growing rate trees, crop residues, industrial wastes, pruning of trees, and as the case under study in this paper, native perennials grasses (Cotana et al., 2014; Sarkar et al., 2012; Sharma et al., 2011; Wang et al., 2012). Within this group of lignocellulosic materials, perennial C4 grasses appear as a promising, yet unexploited alternative presenting the following advantages: (i) high growth rates; (ii) can be grown in marginal areas not suitable for agriculture; (iii) low inputs requirements; (iv) high water and nitrogen use efficiency, and (v) higher net energy ratio and less negative environmental impacts compared with first generation biofuels (Karp and Shield, 2008).

Spartina argentinensis Parodi is the dominant species of an area that occupies *circa* 33,500 km^2 in Argentina. Soils in this region are poorly drained and present high salinity resulting unsuitable for crop production. This species has high CO_2 fixation rates and its growth rate increases after a disturbance such as fire or removal of the aboveground biomass (Feldman et al., 2004).

Due to the low digestibility of *S. argentinensis*, its dry matter accumulates without being grazed. Afterwards, prescribed fire is commonly performed by farmers aiming to stimulate better digestibility forage for their cattle (Jozami et al., 2013). Low stocking rates and hence low annual production of beef per ha are common results of these grasslands. Moreover, such ecosystems are a huge source of biomass for which, so far, no sustainable productive activities have been either proposed to or adopted by farmers. Its use for bioethanol production would face no conflicts regarding competition for food crops or for land use changes. Moreover, there is evidence that indicates that net CO_2 emissions following the use of lignocellulosic bioethanol would likely be much lower than those derived from fossil fuels (Don et al., 2012; Felten et al., 2013), hence meeting the needs to mitigate the greenhouse effect (Qin et al., 2014).

Although there is much literature pointing out lignocellulosic bioethanol as a clean and "environmentally friendly" energy (Nigam and Singh, 2010), there is still one structural barrier to be overcome in order to make viable its production. This barrier consists of the lignin polymer that limits the accessibility to cellulose and hemicellulose polysaccharides by hydrolytic enzymes. Thus, before hydrolysis, biomass must be mandatorily pretreated in order to open the lignocellulose matrix, exposing its structural polysaccharides to the action of hydrolytic enzymes. Active research is on worldwide to evaluate different processes such as ammonium, acid, different alkalis or other pretreatments. However, these procedures, carried out at an industrial scale would generate many negative environmental impacts. Pretreatments that subject the biomass to high temperatures are highly energy demanding and affect negatively the overall energetic balance. Hence, some authors claim that biological pretreatments such as lignin degrading enzymes, or fungal extracts that naturally produce such enzymes could be an effective, environmentally friendly method to overcome the lignin barrier for second generation bioethanol with low energy requirements (Dashtban et al., 2009; Hamelinck et al., 2005; Nigam and Singh, 2010; Wang et al., 2013). However, many authors claim that biological pretreatments are slow, require sterile conditions, present low yields and in many cases, some fungus hydrolyze a significant part of the cellulose (Keshwani and Cheng, 2009; Menon and Rao, 2012; Sun and Cheng, 2002).

In the present study, a 2-day pretreatment with a supernatant of *Pycnoporus sanguineus* triggered a glucose yield of 56.84% of cellulose available in senescent leaves of *S. argentinensis*, with no comparable values using commercial ligninolytic enzymes or phosphoric acid as pretreatment agents. Those results suggest that *S. argentinensis* can become a suitable substrate for second generation bioethanol production employing a short and non contaminating biological pretreatment.

2. Methods

2.1. Biomass, chemicals, and enzymes

S. argentinensis Parodi plants were originally transplanted from a watershed halophyte grassland community of the depressed area surrounding river Ludueña (33°S; 60°; 50′W) to the Experimental Campus of the Facultad de Ciencias Agrarias from the Universidad Nacional de Rosario, Zavalla (33° 01′S; 60° 53′°W). Leaves were harvested, oven dried to constant weight (60 °C – 48 h), ground to 3 × 2 × 1 mm pieces, and stored in plastic bags at room temperature. Senescent leaves can remain attached to the plant for long periods (S. Feldman, personal communication) and can constitute a significant amount of biomass, 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), hemicellulase from *Aspergillus niger* (H2125), cellulase from *A. niger* (C1184), lignin peroxidase (42,603), manganese peroxidase (93,014), and *Trametes versicolor* laccase (51,639).

2.2. Fungal species for biomass pretreatments

Two white rot fungi were used in this work: *Trametes hirsuta* (Wulfen) Pilát, and *P. sanguineus* (Fr.) Murr. The fungal basidiocarps were collected from different trees at the Experimental Campus, Zavalla. Fungi were isolated by extracting mycelia from basidiocarps in laminar flow chamber and inoculated in Petri dishes with potato dextrose agar solid medium for incubation during 7 days in oven at 28 °C and dark. Inocula were kept at 4 °C and subcultured periodically.

2.3. Chemical composition of biomass

Chemical composition (percentage of cellulose, hemicellulose and lignin) of green and senescent biomass from *S. argentinensis* was determined by the detergent system used to evaluate the nutritional value of foods for ruminants (Van Soest and Wine, 1967).

2.4. Chemical biomass pretreatment

Fifteen mg of ground dried green and senescent leaves of *S. argentinensis* were placed in Eppendorf tubes ($n = 3$) with 500 μL of 85% v/v H_3PO_4. Controls consisted in reaction tubes containing distilled water instead of H_3PO_4. Tubes were placed in an oven at 50 °C for 45 min., centrifuged for 2 min. at 2200*g*, and the supernatant was removed. Phosphoric acid-dissolved cellulose was reconstituted by washing 3 times with 1 mL of cold acetone, and centrifuged at 2200*g* for 5 min. Subsequently, 3 washes with 350 μL of distilled water were performed to remove acetone, centrifuging 5 min. at 2200*g* between washes. Supernatants were saved and pooled to check the presence of fermentable sugars released from the solid phase.

2.5. Biological biomass pretreatment with fungal supernatants

A disk with a diameter of 0.5 cm from each fungus mycelia on YPD plates was inoculated into 30 mL of YPD liquid medium and incubated at 28 °C for 7 days. Supernatant and mycelium of each fungus were separately recovered under sterile conditions. Total

protein concentration was measured (Bradford, 1976) in the fungi supernatants. Tubes containing 15 mg of ground dried green or senescent leaves of *S. argentinensis* were incubated with 1 mL of supernatant of the different fungus growth medium. Control tubes containing the boiled supernatant (denatured enzymes) were also prepared.

For *T. hirsuta*, another experiment was conducted adding 2 mg of mycelium diluted in 3 mL of Na Acetate pH 4 or 3 mL of 1/10 diluted supernatant to flasks containing 50 mg of *S. argentinensis* ground dried green leaves in order to analyze glucose release over time. Also, fresh and frozen supernatant of *T. hirsuta* were used in a pretreatment to evaluate enzyme stability upon storage. Flasks and tubes were incubated at 28 °C. For tubes, the cellulolytic enzyme cocktail (see Section 2.7) was added after 2 days. For flasks, a 10 μL aliquot of the medium was taken every day to monitor glucose release, and after 7 days, the cellulolytic cocktail was added. All tubes and flasks assays were prepared in triplicate.

2.6. Biological biomass pretreatment with commercial enzymes

Fifteen mg of ground dried green or senescent leaves of *S. argentinensis* were placed in Eppendorf tubes ($n = 3$), and 0.005 U of the corresponding ligninolytic enzymes – diluted in 500 μL of 50 mM Na Acetate pH 4.0 – were added. Enzymes were evaluated individually and in every possible combination. Laccase was first evaluated in contrast to phosphoric acid pretreatments using 0.06 U per tube. Controls consisted of reaction tubes containing only buffer. Tubes were incubated at 37 °C for 48 h. Subsequently, samples were subjected to saccharification. To evaluate the progression of the enzymatic pretreatments, a sample was incubated with laccase and aliquots were taken at fixed intervals, upon which they were boiled for 10 min to stop the reaction. All aliquots were subjected to saccharification as described below.

2.7. Biomass saccharification

To investigate the efficiency of cellulolytic enzymes, green or senescent pretreated leaves were incubated in 1.5 mL Eppendorf tubes with a mixture of cellulase, hemicellulase and ß-glucosidase. Each enzyme (0.4 U) was dissolved in 500 μL of 100 mM Na Acetate pH 6.0 immediately prior to use. Subsequently, tubes were incubated at 50 °C for 48 h, cooled to room temperature and glucose concentration measured by the glucose oxidase method.

Tubes with saccharified biomass were centrifuged at 1500*g* for 5 min., and 10 μL aliquots were withdrawn for glucose determination with a commercial enzymatic glycemia kit (Wiener Lab, Rosario, Argentina). Tubes were incubated at 35 °C for 15 min., and then absorbance at 505 nm was measured in a spectrophotometer (Perkin Elmer Lambda Bio+). Glucose concentration in each tube was calculated by constructing a calibration curve built with a glucose solution of known concentration (1 g/L). Blanks were prepared in every experiment to account for the presence of added sugars to the commercial preparations. Values of released glucose of these controls were subtracted from those of pretreated or control saccharified biomass. Results were expressed as mg of glucose released per g of *S. argentinensis*.

Percentage of hydrolysed cellulose was calculated (omitting the negligible glucose from the hydrolysis of hemicelluloses) as follows:

$$\%\ \text{hydrolysed cellulose} = (\text{mg of released glucose per g of } S.argentinensis \times 0.9^{*}/\text{mg of cellulose per g of } S.argentinensis) \times 100$$

*0.9 is a correction factor considers that each glucose moiety in the cellulose chain weights 162 Da, while released glucose weights 180 Da.

2.8. Enzyme filtering

Sugars present as stabilizers in commercial cellulolytic enzymes were removed according to Penefsky (1977), using 5 ml columns equilibrated in Hepes–NaOH, 5 mM $MgCl_2$ and 10% (v/v) glycerol, pH 7.0. Cellulases were recovered in Eppendorf tubes, which were stored at −20 °C.

2.9. Statistical analysis of the results

Data were analyzed using the statistical software InfoStat (Di Rienzo et al., 2014).

3. Results and discussion

3.1. Chemical composition of S. argentinensis biomass

The percentages of different components of *S. argentinensis* biomass according to Van Soest assay are shown in Table 1. Polysaccharides accounts for almost 70% of dry matter (cellulose: 38–47%; hemicellulose: 23–32%), offering an interesting potential for bioethanol production. Lignin contents are close to 7%. Jozami et al. (2013) calculated that between 1690 and 2800 L/ha of bioethanol could be produced from *S. argentinensis*, considering hydrolytic efficiencies of 60% and 90%, respectively. It's interesting to note that a significant difference in the content of each polysaccharide between green and senescent leaves was found (32% vs. 23% for hemicellulose content, respectively, and 38% vs. 47% for cellulose content).

3.2. Pretreatments with P. sanguineus and T. hirsuta supernatants

P. sanguineus supernatant pretreatment of ground leaves showed a higher amount of sugar released compared to *T. hirsuta* after the addition of cellulolytic enzymes (Fig. 1), and differed according to the type of leaves used (p-value = 0.0001), being significantly higher for senescent than for green leaves (offering the possibility to harvest the grass once its life cycle is complete). The latter difference could be partially explained by the chemical composition of both types of biomass, since senescent leaves have more cellulose and less hemicellulose (Table 1). Further studies should be carried out to find out if structural features can also account for the effect observed.

Both fungi supernatant were assayed globally and not according to specific ligninolytic activities. Protein concentration in fungi supernatants were different, 1.72 μg/mL for *P. sanguineus* and 2.32 μg/mL for *T. hirsuta*. In the pretreatment with *P. sanguineus* supernatant, glucose release reached a value close to 300 mg per g of senescent leaf tissue, which corresponds to approximately 38% of total carbohydrates, and 56.8% of plant cellulose (Fig. 1a). When data are normalized according to the protein present in the extracts, differences in favor of *P. sanguineus* became more conspicuous (Fig. 1b). This disparity could be accounted for either a different composition of the fungal supernatant or a different

Table 1
Chemical composition of *Spartina argentinensis* biomass, expressed as percentages.

Leaves	Hemicellulose	Cellulose	Lignin
Green	32.3	37.91	7.22
Senescent	23.17	47.5	7.89

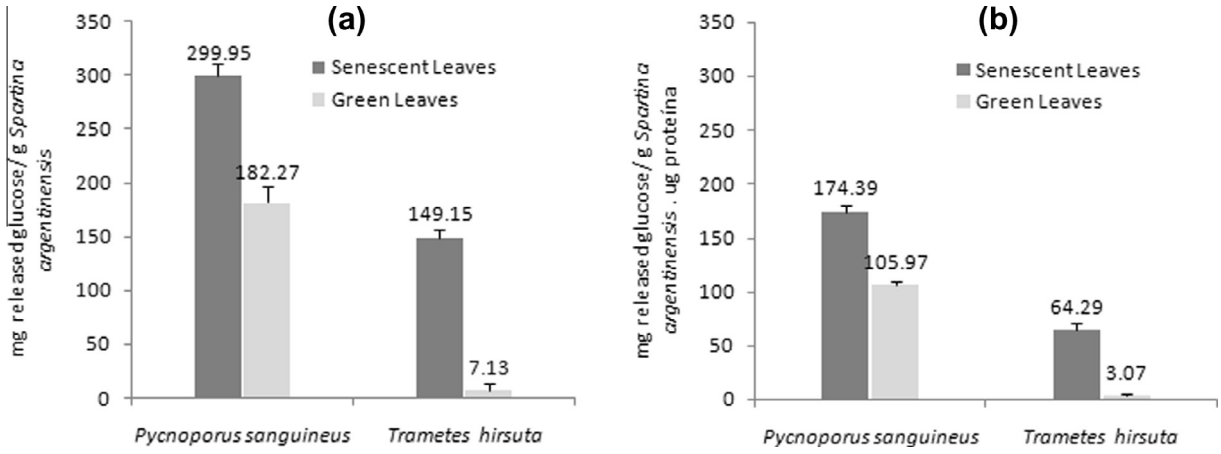

Fig. 1. Released glucose after a 2-day pretreatment with the supernatant of *P. sanguineus* and *T. hirsuta* fungi on green and senescent leaves of *S. argentinensis*, followed by a 3-day enzymatic hydrolysis step ($n = 3$), under not normalized (a) and normalized (b) data.

lifetime of the enzymes in each, or both factors. These results are promising taking into account the short period of fungal incubation (7 days in YPD medium) and pretreatment (2 days) when comparing them with other successful biological pretreatments where incubation of 4–6 weeks were necessary (Liu et al., 2013; Sarkar et al., 2012; Sun and Cheng, 2002). Liu et al. (2013), evaluated a strain of *P. sanguineus* as a biological pretreatment in *Panicum prionitis* (Switchgrass) obtaining a 90% of glucan hydrolysis after cellulolytic digestion. Regardless of the differences in the type of biomass, the incubation (36 vs. 7 days) and enzymatic hydrolysis (5 vs. 2 days) periods were longer than in the experiments described here.

For *T. hirsuta* there was no significant glucose release in senescent leaves after pretreatment (Fig. 1a). Furthermore, fresh supernatant showed a release of *circa* 150 mg glucose/g of *S. argentinensis* (Fig. 1a), while frozen supernatant was significantly less efficient, triggering 49.26 mg glucose/g of biomass (p-value = 0.0004, data not shown).

Pretreatment with *T. hirsuta* mycelium in flasks showed that released glucose increases up to the third day of co-culture with the plant biomass, reaching a value of almost 20 mg glucose/g *S. argentinensis,* falling then even after the addition of cellulases (Fig. 2). However, the behavior of fungal supernatant differed from that of fungal mycelium. In this case, released glucose values remained low until the seventh day, when the addition of the cellulolytic cocktail triggered sugar release, reaching a maximum value near 120 mg glucose/g *S. argentinensis* (Fig. 2).

Values for saccharified but not pretreated biomass never exceeded those of the control values. Since no glucose was produced in non-pretreated biomass, then pretreatments are the sole responsible for glucose release.

3.3. Ligninolytic enzyme pretreatments

Pretreatments involving commercial ligninolytic enzymes resulted almost ten-fold less effective than the fungal supernatant pretreatments. Laccase pretreatment produced a higher release of glucose than pretreatment with 85% v/v phosphoric acid, whereas a sequential pretreatment resulted in lower yields (Fig. 3), probably due to enzyme inhibition by reaction products of the first step. Yields were lower for senescent leaves in all cases (Fig. 3). Differences between all pretreatments were significant (p-values < 0.05). Plácido et al. (2013), obtained lower yields with a commercial laccase used as a pretreatment in cotton gin trash.

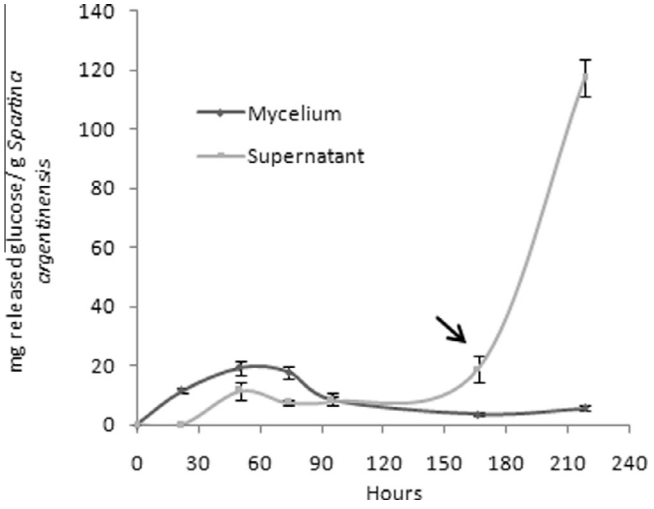

Fig. 2. Released glucose during pretreatment with the mycelium and 1/10 diluted supernatant of *T. hirsuta* on green leaves of *S. argentinensis*, with stirring. The error bars represent the standard deviation ($n = 3$). Arrow marks the addition of cellulolytic enzymes the 7th day.

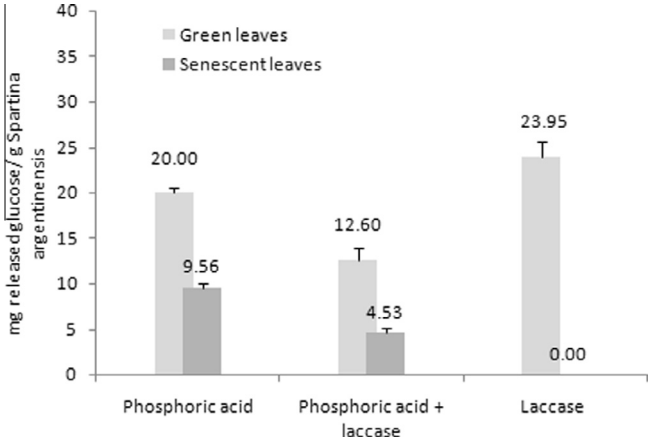

Fig. 3. Released glucose during enzymatic hydrolysis, previous pretreatment with H_3PO_4 and laccase on green and senescent leaves of *S. argentinensis*. The error bars represent the standard deviation.

LiP and MnP pretreatments resulted in greater glucose release than laccase pretreatment. MnP showed a slightly higher yield than LiP, but differences were not significant (Fig. 4). Sequential pretreatments with 85% v/v phosphoric acid and enzymes resulted in less release of sugars for all the enzymes (data not shown). Reducing laccase concentration by 92% caused a 66.5% yield decrease, so there was no linear relationship between these two variables within the range of enzyme concentrations used in these experiments.

As shown in Fig. 4, pretreatment with the three enzymes increased glucose release compared to single pretreatment with either one. However, neither an additive nor a synergistic effect was observed in the action of these enzymes, probably because enzyme concentrations were already high enough to produce maximum hydrolysis. Pretreatments with two enzymes had similar yields regardless the combination used, and very close to that achieved by combination of the three enzymes. There were no significant differences between these four pretreatments (p-values > 0.05).

No release of glucose was observed in senescent leaves, in any of the enzyme pretreatments assayed. Although the efficiency of glucose liberation from biomass with commercial enzymes is lower than that observed in experiments with fungal supernatants, an objective comparison cannot be made in the light of the fact that ligninolysis with the latter procedure is probably being exerted by a consortia of enzymes and low molecular weight oxidant compounds (Salvachúa et al., 2011).

3.4. Time course of laccase action

A one day-pretreatment was similar to that produced by two days with the same amount of enzyme (compare Figs. 5 and 3). It is Interesting to note that, in this case, there was a large release of glucose using senescent leaves. A possible reason for the difference could lie in that heat treatment affects in different way both types of leaves, producing an increased enzymatic accessibility for naturally senescent leaves. These results suggest a structural difference between the two types of leaves, as does the fact that the enzyme pretreatments without boiling are effective on green, but not on senescent leaves.

3.5. Comparison of released cellulose in all pretreatments

Table 2 shows the percentage of released cellulose from *S. argentinensis* biomass analyzing and comparing the results

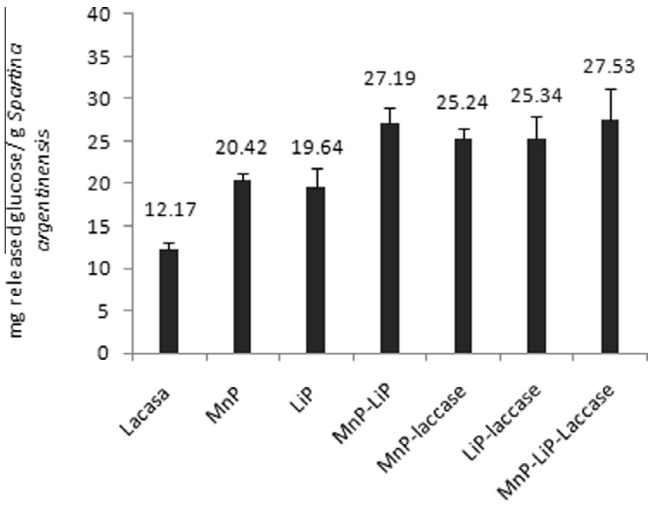

Fig. 4. Released glucose during enzymatic hydrolysis, previous pretreatment with each ligninolytic enzyme and all the possible combinations of them on green leaves of *S. argentinensis* ($n = 3$). The error bars represent the standard deviation.

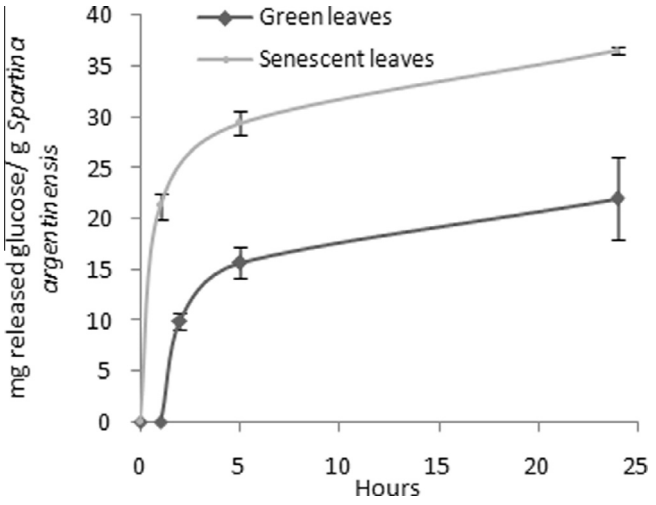

Fig. 5. Released glucose during enzymatic hydrolysis, previous laccase pretreatments of different duration on green and senescent leaves of *S. argentinensis*. The error bars represent the standard deviation.

obtained with different pretreatments assayed and described previously. Thus, it can be said that the supernatant of *P. sanguineus* culture is the best pretreatment for lignin degradation of *S. argentinensis* biomass. Although it should be considered that incubation time was quite longer for this pretreatment compared to those using commercial ligninolytic enzymes, the latter were more expensive and less effective. It may well be that ligninolytic fungi have a complex system consisting of a wide variety of enzymes that act synergistically and sequentially, making it more efficient than a ligninolytic cocktail. Further efforts need to be done to characterize the enzyme composition of these supernatants.

Theoretical yields in bioethanol for different crops are summarized in Table 3, and the composition of the biomass in each case is indicated. It should be noted that although food crops have generally a lower lignin content, which increases ethanol yield, promising values are observed for *Miscanthus*, *switchgrass* and *Spartina*. *S. argentinensis* has a significant potential for the intended purpose, with yields that could reach 3900 L/ha, while the equivalent value for a consolidated crop as corn would be around 3700 L/ha. Besides, at similar bioethanol yields, *S. argentinensis* offers the additional advantage of being a rangeland pasture not used for food production, does not need an intensive agriculture management as a bioenergy crop does (i.e., it does not alter the land use, it does not have any cost for sowing, fertilization, spraying, etc),

Table 2
Fraction of released cellulose for all pretreatments.

Pretreatment	% Of hydrolysed cellulose	Biomass leaves	Total time invested (d)
H_3PO_4	4.74	Green	3
H_3PO_4 + laccase 0.06 U/tube	2.99	Green	5
Laccase 0.06 U/tube	5.69	Green	5
Laccase 0.005 U/tube	2.89	Green	5
MnP 0.005 U/tube	4.85	Green	5
LiP 0.005 U/tube	4.66	Green	5
MnP–LiP–laccase	6.53	Green	5
MnP–LiP	6.45	Green	5
MnP–laccase	5.99	Green	5
LiP–laccase	6.01	Green	5
Trametes hirsuta supernatant	27.85	Green	13
P. sanguineus supernatant	56.84	Senescent	13

Table 3
Composition and yields of different biomass into bioethanol. t/ha refers to tonnes of dry matter per hectare.

Biomass	% Cellulose	% Hemicellulose	% Lignin	Average biomass yield	Bioethanol yield
Corn	35[2]	28[2]	10.4[2]	9.1–20 t/ha[8,18]	3725–6890 L/ha[8]
Sugarcane	24[2]	8[2]	7[2]	10.4–17.4 t/ha[2]	7000 L/ha[2]
Miscanthus	38.2–57.6[2,5]	15.9–24.3[2,5]	10.5–24.1[2,5]	3–35.76 t/ha[8,18]	1030–13,770 L/ha[8]
Switchgrass	31.6–45[2,19]	31.4–36[2,19]	6.1–12[2,19]	3–21 t/ha[19]	1030–7230 L/ha[8]
Spartina	37.91–47.5	23.17–32.3	7.22–7.89	10 t/ha[12]	2348–3913 L/ha[15]

and contributes to a more sustainable biofuel production system. Finally, a new biofuel industry based on this feedstock located in its natural growing area could positively affect the social and economic conditions of the rural population, by generating new sources of employment and, consequently, allowing the regional development.

4. Conclusions

As a conclusion of this work, we show that *S. argentinensis*, an extensive rangeland grass with little value as pasture may be considered an interesting feedstock for second generation bioethanol. Ligninolysis with a fungal supernatant allowed to reach near 60% of glucose release for fermentation after a short (2 days) pretreatment. Further research is needed to optimize the saccharification process and transfer these results to other similar feedstocks.

Acknowledgements

This work was supported with funds from Proyecto de Vinculación Tecnológica "Ingenierio Enrique Mosconi" Ministerio de Educación Nacional, Secretaría de Ciencia, Tecnología e Innovación Productiva de la Provincia de Santa Fe (Argentina), and Fundación Nuevo Banco de Santa Fe. F.E.P. and S.R.F. are career members of CONICET and CIUNR, respectively.

References

Bradford, M.M., 1976. Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Cotana, F., Cavalaglio, G., Gelosia, M., Nicolini, A., Coccia, V., Petrozzi, A., 2014. Production of bioethanol in a second generation prototype from pine wood chips. Energy Procedia 45, 42–51.

Dashtban, M., Schraft, H., Qin, W., 2009. Fungal bioconversion of lignocellulosic residues; opportunities & perspectives. Int. J. Biol. Sci. 5, 578–595.

Di Rienzo, J.A., Casanoves, F., Balzarini, M.G., Gonzalez, L., Tablada, M., Robledo, C.W., 2014. InfoStat, 2014 version. InfoStat Group, FCA, National University of Córdoba, Argentina. URL <http://www.infostat.com.ar>.

Don, A., Osborne, B., Hastings, A., Skiba, U., Carter, M.S., Drewer, J., Flessa, H., Freibauer, A., Hyvönen, N., Jones, M.B., Lanigan, G.J., Mander, Ü., Monti, A., Djomo, S.N., Valentine, J., Walter, K., Zegada-Lizarazu, W., Zenone, T., 2012. Land-use change to bioenergy production in Europe: implications for the greenhouse gas balance and soil carbon. Glob. Change Biol. Bioenergy 4, 372–391.

FAO, 2008. The State of Food and Agriculture 2008, Biofuels: prospects, risks and opportunities. Available at <http://www.fao.org/bioenergy/1628901af23124a8c86ce2180954a8a88b96f1.pdf. Last accessed on 15/4/15>.

Feldman, S.R., Bisaro, V., Lewis, J.P., 2004. Photosynthetic and growth responses to fire of the subtropical-temperate grass *Spartina argentinensis* Parodi. Flora 199, 491–499.

Felten, D., Fröba, N., Fries, J., Emmerling, C., 2013. Energy balances and greenhouse gas-mitigation potentials of bioenergy cropping systems (Miscanthus, rapeseed, and maize) based on farming conditions in Western Germany. Renew. Energy 55, 160–174.

Hamelinck, C.N., Van Hooijdonk, G., Faaij, A.P., 2005. Ethanol from lignocellulosic biomass: techno-economic performance in short-, middle-and long-term. Biomass Bioenergy 28, 384–410.

IEO (International Energy Outlook), 2010. DOE/EIA-0484, U.S. Department of Energy, Energy Information Administration, Washington, DC, available at <http://www.eia.gov/oiaf/aeo/pdf/0383(2010).pdf>. Last accessed on 2/4/15.

Jozami, E., Sosa, L.L., Feldman, S.R., 2013. *Spartina argentinensis* as feedstock for bioethanol. Appl. Technol. Innov. 9, 37–44.

Karp, A., Shield, I., 2008. Bioenergy from plants and the sustainable yield challenge. New Phytol. 179, 15–32.

Keshwani, D.R., Cheng, J.J., 2009. Switchgrass for bioethanol and other value-added applications: a review. Bioresour. Technol. 100, 1515–1523.

Langeveld, J.W., Dixon, J., van Keulen, H., Quist-Wessel, P.M., 2014. Analyzing the effect of biofuel expansion on land use in major producing countries: evidence of increased multiple cropping. Biofuels Bioprod. Bioref. 8, 49–58.

Liu, J., Wang, M.L., Tonnis, B., Habteselassie, M., Liao, X., Huang, Q., 2013. Fungal pretreatment of switchgrass for improved saccharification and simultaneous enzyme production. Bioresour. Technol. 135, 39–45.

Menon, V., Rao, M., 2012. Trends in bioconversion of lignocellulose: biofuels, platform chemicals & biorefinery concept. Prog. Energy Combust. Sci. 38, 522–550.

Nigam, P.S., Singh, A., 2010. Production of liquid biofuels from renewable resources. Prog. Energy Combust. Sci. 37, 1–17.

Penefsky, H.S., 1977. Reversible binding of Pi by beef heart mitochondrial adenosine triphosphatase. J. Biol. Chem. 252, 2891–2899.

Plácido, J., Imam, T., Capareda, S., 2013. Evaluation of ligninolytic enzymes, ultrasonication and liquid hot water as pretreatments for bioethanol production from cotton gin trash. Bioresour. Technol. 139, 203–208.

Qin, D., Plattner, G.K., Tignor, M., Allen, S.K., Boschung, J., Nauels, A., Midgley, P.M., 2014. In: Stocker, T. (Ed.), Climate Change 2013: The Physical Science Basis. Cambridge University Press, Cambridge, UK, and New York.

Rajagopal, D., Zilberman, D., 2007. Review of Environmental, Economic and Policy Aspects of Biofuels, vol. 4341. World Bank Publications.

Salvachúa, D., Prieto, A., López-Abelairas, M., Lu-Chau, T., Martínez, Á.T., Martínez, M.J., 2011. Fungal pretreatment: an alternative in second-generation ethanol from wheat straw. Bioresour. Technol. 102, 7500–7506.

Sarkar, N., Ghosh, S.K., Bannerjee, S., Aikat, K., 2012. Bioethanol production from agricultural wastes: an overview. Renew. Energy 37, 19–27.

Sharma, H.S.S., Lyons, G., McRoberts, C., 2011. Biorefining of perennial grasses: a potential sustainable option for Northern Ireland grassland production. Chem. Eng. Res. Des. 89, 2309–2321.

Solomon, B.D., Bares, J.R., Halvorsen, K.E., 2007. Grain and cellulosic ethanol: history, economics, and energy policy. Biomass Bioenergy 31, 416–425.

Sun, Y., Cheng, J., 2002. Hydrolysis of lignocellulosic materials for ethanol production: a review. Bioresour. Technol. 83, 1–11.

Van Soest, P.J., Wine, R.H., 1967. Use of detergents in the analysis of fibrous feed. IV. The determination of plant cell wall constituents. J. Assoc. Off. Anal. Chem. 46, 825–835.

Wang, S., Hastings, A., Smith, P., 2012. An optimization model for energy crop supply. Glob. Change Biol. Bioenergy 4, 88–95.

Wang, F.Q., Xie, H., Chen, W., Wang, E.T., Du, F.G., Song, A.D., 2013. Biological pretreatment of corn stover with ligninolytic enzyme for high efficient enzymatic hydrolysis. Bioresour. Technol. 144, 572–578.