Biodegradation Pretreatment of Wood of *E. grandis*, *E. dunnii*, and *E. benthamii* to Work in Biorefinery Processes

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ABSTRACT: Nowadays, there is a great interest in using lignocellulosic materials as substrate for the production of biorefinery products. *Eucalypti* are good options to use as crops to obtain different kinds of biofuels and derivatives, since their plantations show high adaptation potential to soil and weather conditions in Uruguay.

The basic process steps involved in the obtainment of biorefinery materials are: pretreatment, hydrolysis, fermentation and products separation. As delignification is an important process to obtain biorefinery products, in this context the evaluation of the biological (BT) and hydrothermal (TT) pretreatment of different species of *Eucaliptus* was studied. The possibility of obtaining sugars, alcohols and organic acids was the main focus.

The results of these investigations show a good production of reducing sugars (4–5 mg/mL for both BT and TT pretreatments), acetic acid (3–8 mg/mL for BT and 3–7 mg/mL for TT) and isopropanol (18–48 mg/mL for BT and 20–30 mg/mL for TT). In conclusion, the results show similar behaviours for BT and TT pretreatments, which is a quite important result since BT is cheaper and cleaner and thus a more attractive technology.

KEYWORDS: Biorefinery, delignification, pretreatment

1 INTRODUCTION

These days there is a great interest in using lignocellulosic materials as substrate for the production of biorefinery products, because they serve as cheap and abundant feedstocks. The three main chemical fractions that constitute lignocellulosic materials are: cellulose, hemicellulose and lignin. These constituents, and their derivatives, allow obtaining products with high added value, and in many aspects follow a scheme similar to oil refining [1, 2]. Cellulose is a natural polymer used daily in the production of paper. It is possible to hydrolyse it to its constituent monomers to obtain fermentable media to produce ethanol, butanol and propanol [3–6].

Hemicelluloses and their derived monomeric sugars are also fermentable media, but in this case, given the wide variety of monomers and oligomers, the possibility of obtaining different chemicals in a broad spectrum occurs. These products can find applications in cosmetics, pharmaceuticals, and dietary and functional foods. Some examples include xylitol, acetic acid, and furfural and derived synthetic polymer products with interesting properties, such as biodegradability, which is an advantage over petroleum-derived plastics. From a chemical point of view, a wide range of chemicals can be obtained from heteropolysaccharides, such as succinic acid, fumaric and malic acids, 2,5 furan dicarboxylic acid, aspartic acid, glutamic acid and levulinic acid. Also, alcohols such as glycerol, sorbitol, xilitol and arabinol can be produced.

On the other hand, the integral exploitation of vegetal species demands the necessary biomass deconstruction. In order to reach the best breaking up of species, it is necessary to choose the best wood species

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that will enable obtaining suitable biomass under sustainable conditions. Within biomass deconstruction procedures, there are different types of hydrolysis and fermentations in order to obtain glucose and ethanol [7]. Until today, only laboratory-scale schemes have been tested, leaving behind the scale-up to commercial scale [8].

The break-up of the main biomass components is a challenge; one of the most common risks being the degradation of its chemical structure. Due to the complexity of its structure, together with hardly labile chemical and physical bonds, it is not possible to achieve a convenient degree of break-up through conventional technologies. One of the most important economic barriers is the high resistance of the lignocellulosic matrix, which hinders an easy degradation [9, 6]. The most important thing to bear in mind is which stages to use in integral break-up and which products should be found. The most promising strategies have been developed in connection with the production of ethanol, an important biocombustible used as fuel for transport [10, 11]. The identification of the best procedures for lignocellulosic biomass is therefore considered one of the most interesting areas of work, research and development. Additionally, many biological and enzymatic methods are known to allow lignin, cellulose and hemicellulose modification, which allow thinking in terms of combined methods to obtain, for example, a variety of phenols and vanillin [1, 12–14].

The aim of this study is to study the biological pretreatment (BT) and hydrothermal pretreatment (TT) of different species of *Eucalyptus* and evaluate the response towards glucose, alcohols and organic acids production.

2 EXPERIMENTAL

2.1 Wood Samples

The wood samples used were *E. benthamii* (B2P7), *E. dunnii* (B1P18), and *E. grandis* (B2P13). They were grown from crops in forest qualified soils in Paysandú (Uruguay). The samples were subjected to biological and hydrothermal treatments and the obtained material tested by hydrolysis and fermentation procedures. All the preformed assays were done in duplicate.

2.2 Biological and Hydrothermal Pretreatments

For the biological pretreatment, 6 Erlenmeyer flasks were prepared with 100 ml of YPD, 100 ml of distilled water and 1 g of wood. Each assay was inoculated with 0.5 ml of *Trametes* and 0.5 ml of *Phanerochaetes* cultures. The pretreatment was maintained during 5 days at 30 °C, and later sterilized in autoclave at 121 °C during 30 min.

For the hydrothermal pretreatment used to improve the recovery of hemicellulose-derived sugars, the wood was impregnated with H_2SO_4 prior to steam pretreatment. Accordingly, the material was impregnated with sulphuric acid solution (2%, v/v) for two hours, filtered and then treated in an autoclave at 121 °C and 1.2 atm pressure for two hours.

2.3 Hydrolysis and Fermentation Procedures

The enzymatic hydrolysis was done with the enzyme in powder form dissolved previously in 0.1 M acetate buffer (pH 4.8) containing 1.1% (w/v) of substrate and 1% (w/v) of the enzyme, respectively (190 kFPU/g dry substrate). The hydrolysis process was conducted during 72 h at 50 °C in a shaker for continuous stirring according to the procedure described in [15]. The sugars obtained during the hydrolysis were thereafter subjected to the fermentation process during 24 h at 30 °C with *S. cerevisidae* culture (V = 30 ml).

2.4 Analysis Methodologies

Fermentable sugars were analysed by the 3,5-dinitrosalicylic acid (DNS) technique. Briefly, 0.25 g of dinitrosalicylic acid and 0.75 g of sodium and potassium tartrate were dissolved in 50 ml of NaOH 2 M and 250 ml of water. Then 1 ml of the sample and 1 ml of DNS were incubated at 100 °C during 10 minutes. The determination was done at 570 nm.

Ethanol and propanol were quantified using gas chromatography-mass spectrometry (GC-MS). For the analysis a gas chromatograph Agilent Model 7890A connected to a mass detector Agilent 5975C inert XL was used. The capillary column was DB-FFAP Agilent 60 m × 0,25 mm × 0.25 µm (film thickness). Injection port temperature was set at 200 °C and oven temperature at 29 °C for 30 minutes. A split ratio 1:100 and a flow rate of carrier gas (helium) of 1 mL/min were used. Interface temperature was 250 °C, source temperature 230 °C and Quadrupole temperature 150 °C. Mass were scanned between 20 and 150 using electron impact at 70 eV as ionization mode.

Organic acids were determined by using a Shimadzu Liquid Chromatograph-Diode Array Detector operating at 210 nm. Two different columns were used: HILIC Zwiterionic (250 mm × 4,6 mm × 5 µm) and a Bio-Rad Aminex 87H (300 mm × 7,8 mm × 9 µm). The mobile phase was acetonitrile:sulphuric acid 10 mMat at different volume ratios for HILIC column, and 100% sulphuric

acid 10 mM for Aminex column. For HILIC column three different column temperatures (25 °C to 50 °C) were used and for Aminex column 50 °C was used.

Fungus development in the wood subjected to biological treatment was determined by scanning electron microscopy (SEM) using an apparatus JEOL 5900LV.

3 RESULTS AND DISCUSSION

3.1 Evaluation of the Biomass Productivity

The growth rates were similar for those of *E. benthamii*, *E. dunnii*, and *E. grandis*, as well as for the biomass productivity, showing that these two parameters are independent of the studied species. The high survival levels, basically caused by good weed control, determined the homogeneous growth, as shown in Figure 1.

3.2 Development of Wood Fungus as Determined by Scanning Electron Microscopy

The analysis performed by SEM shows that *Phanerochaetes* and *Trametes* consortium presented a good development, as corroborated by the SEM analysis presented in Figure 2. The growth of the consortium was successful in all studied wood species.

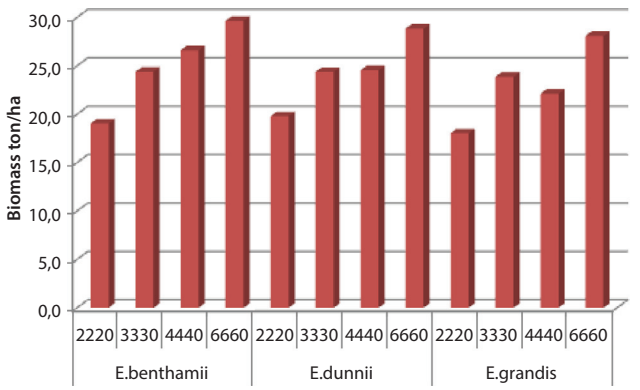

Figure 1 Growth profile of the three studied species, showing a homogeneous pattern.

3.3 Reducing Sugar Production

The reducing sugars content obtained with the three studied species and the two pretreatments (BT and TT) was very similar. The obtained results are shown in Table 1.

3.4 Identification and Quantification of Alcohols by GC-MS

The isopropanol was identified based on the mass spectrum (Figure 3), and its concentration in the analysed samples was quantified by using a calibration curve of external standards in the range of 10 to 80 mg/mL. A good linearity between the isopropanol concentration and respective peak area was obtained ($r^2 = 0.999$). Ethanol and other alcohols were identified following the same analytical conditions used.

From the results shown in Table 2, and comparatively with the other species, *E. grandis* give rise to the highest value of isopropanol in both pretreatments, the one of BT being greater than the one of TT. Within the experimental conditions used, isopropanol was the only detected and quantified alcohol (ethanol was not detected).

In conclusion, the method used for the determination of alcohols was rapid and sensitive. It allowed determining the presence and absence of various alcohols. Future studies can also explore the presence of other volatile compounds formed during the fermentation that are of industrial importance.

3.5 Organic Acids Analysis by HPLC

The results for acetic acid are shown in Table 3 and Figure 4. Formic acid, propionic acid and butyric acid were not detected. Acetic acid was identified in all the

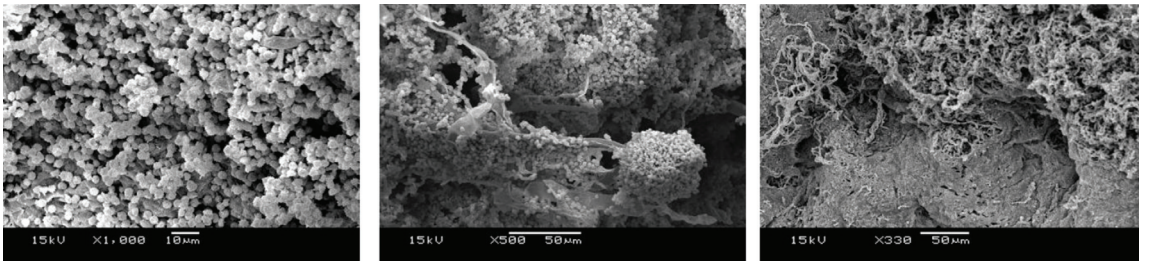

Figure 2 Scanning electron microscopy for the consortium constituted by the fungus *Phanerochaetes* and *Trametes* growing, from left to right, in *E. benthamii*, *E. grandis*, and *E. dunii*.

Table 1 Obtained reducing sugar concentration as a function of the treated species and type of treatment.

Treatment	Sample	Reducing Sugars (mg/mL)
BT	*E. dunii*	5
BT	*E. benthamii*	4
BT	*E. grandis*	5
TT	*E. dunii*	5
TT	*E- benthamii*	4
TT	*E. grandis*	5

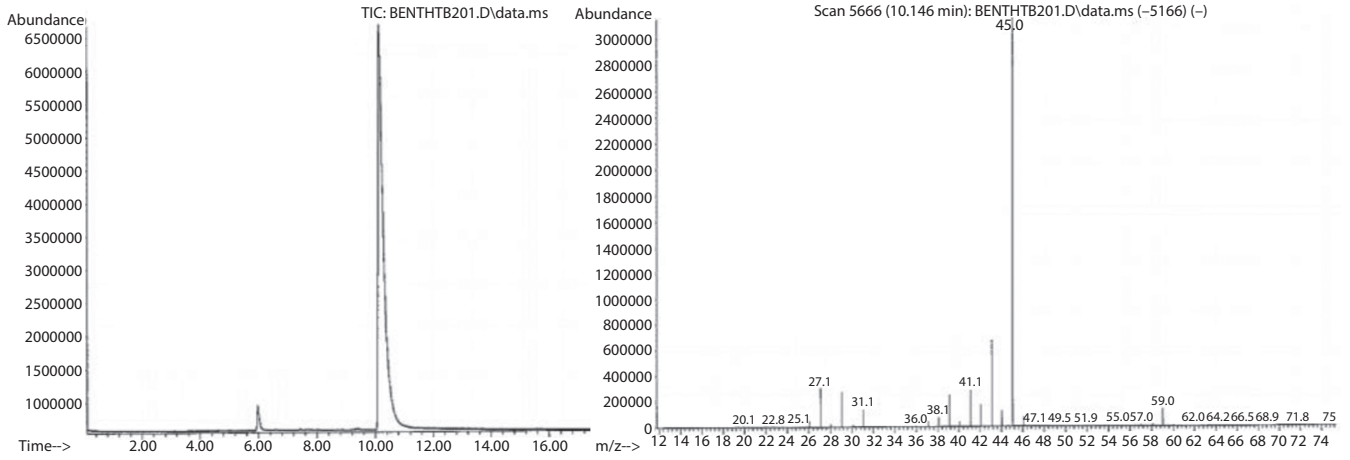

Figure 3 Mass chromatogram (left graph) and Mass spectrum (right graph).

Table 2 Isopropanol concentration obtained after hydrolysis and fermentation of different *Eucalyptus* species.

Type of pre-treatment	Sample	Isopropanol (mg/mL)
BT	*E. dunii*	18
BT	*E. benthamii*	28
BT	*E. grandis*	48
TT	*E. dunii*	21
TT	*E- benthamii*	20
TT	*E. grandis*	30

Table 3 Acetic acid concentration obtained after hydrolysis and fermentation of the different *Eucalyptus* species.

	Sample	Acetic acid (mg/mL)
BT	*E. dunii*	3
BT	*E. benthamii*	5
BT	*E. grandis*	8
TT	*E. dunii*	3
TT	*E- benthamii*	3
TT	*E. grandis*	7

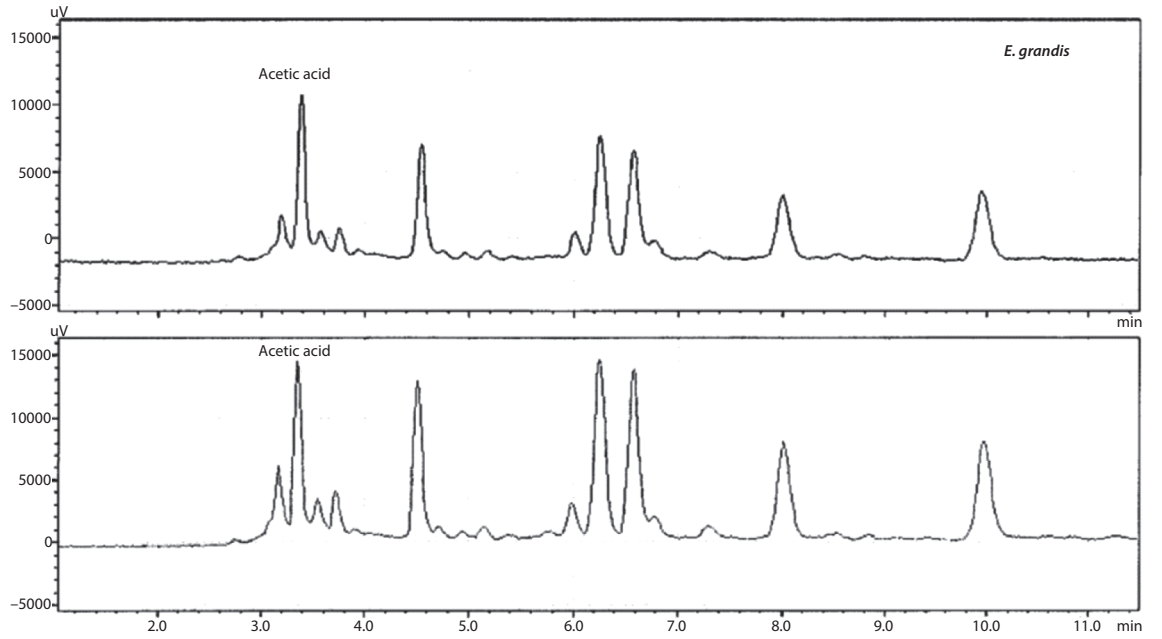

Figure 4 Biological pretreatment (above) and hydrothermal pretreatment (below) for *E. grandis*.

species and in both pretreatments. The highest concentration was found in *E. grandis*.

4 CONCLUSIONS

The growth rates and the biomass productivity were similar for *E. grandis*, *E. dunnii*, and *E. benthamii*, showing that these two parameters are independent for the studied species.

For all the cases, the biological pretreatment gives rise to similar results comparatively with the chemical pretreatment. This is an important feature since the biological pretreatment can be considered a clean and environmentally friendly treatment. Moreover, the hydrothermal treatment is expensive and promotes the production of compounds that can act as inhibitors for the fermentation step. In conclusion, the biological treatment is by far the more attractive option.

The hydrothermal and biological pretreatments lead to similar results in terms of fermentable sugars production, compounds which can act as precursors of high-added-value chemicals, such as acids and alcohols, and polymeric materials.

Regarding the identification of the produced chemicals, in both processes alcohols, such as isopropanol, were detected. It is important to mention that this result may be related to the long-lasting process of fermentation; originally done to observe the remaining present alcohols. These results are valid for both pretreatments, being more efficient in the case where the biological pretreatment was used. Regarding acids production, the most common one was acetic acid, this fact being reported in other published works. The achieved results were also similar for both pretreatment types, again corroborating the advantages of the biological pretreatment.

This is an ongoing work and future tasks will consider the kinetics involved in the production of these chemicals.

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