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Bioethanol from the Portuguese forest residue *Pterospartum tridentatum* – An evaluation of pretreatment strategy for enzymatic saccharification and sugars fermentation

S. Ferreira [a], N. Gil [a], J.A. Queiroz [a,b], A.P. Duarte [a,b], F.C. Domingues [a,b,*]

[a] *Research Unit of Textile and Paper Materials, University of Beira Interior, Av. Marquês D' Ávila e Bolama, 6201-001 Covilhã, Portugal*
[b] *CICS – Health Sciences Research Centre, University of Beira Interior, Av. Infante D. Henrique, 6201-556 Covilhã, Portugal*

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ABSTRACT

Under the current energy scenario, the development of alternatives to fossil fuels, like bioethanol from lignocellulosic materials, is highly relevant. Therefore it is important to search and study new raw materials and to optimize the different steps that lead to bioethanol production. In this work, acid diluted pretreatment was optimized considering the release of sugars. Under the optimal conditions, the reducing sugars yield was of 293.4 mg/g of dry biomass in liquid fraction. The tested pretreated samples of *Pterospartum tridentatum* that presented a higher glucose yield in enzymatic saccharification where those that were subject to a pretreatment at 180 °C for 75 min with 2.75% (w/w) of sulfuric acid when using a biomass/liquid ratio of 2.25 g/10 mL leading to a maximum yield of glucose that was 92% of the theoretical maximum. From the fermentation of filtrates it was possible to obtain a maximum ethanol yield of 0.26 g ethanol/g total sugars, without previous detoxification.

1. Introduction

Alternatives to petroleum-derived fuels are being sought in order to reduce the world's dependence on non-renewable resources. The most common renewable fuel today is ethanol derived from corn grain (starch) and sugar cane (sucrose); however, it is expected that there will be limits to the supply of these raw materials in the near future and the reduction of greenhouse gases resulting from use of sugar or starch-based ethanol is not as high as desirable. Consequently, future large-scale use of ethanol will most certainly have to be based on production from lignocellulosic materials (Hahn-Hagerdal et al., 2006).

Cellulose rich lignocellulosic biomass, such as agricultural residues, forestry residues, wood and energy crops, is an attractive material for bioethanol fuel production since it is the most abundant reproducible resource on Earth (Balat et al., 2008). Lignocellulosic biomass, mainly composed by cellulose, hemicelluloses and lignin, is a cheap substrate, which could make bioethanol more competitive against fossil fuels. In addition, it avoids ethical concerns associated with the use of potential food resources.

The Portuguese forest occupies 3.4 million hectares, i.e. 38.4% of the territory, with 2 million hectares of shrubs presents in native forest and in fallow land, it can be seen as potential source of large amounts of forestry residues, including trees branches, pruning residues and shrubs (CELPA, 2007). The fact that scrubs lands cover large areas of land (in the Iberian Peninsula is estimated an area of approximately 10 million hectares of scrubland) could encourage the use of this feedstock for ethanol production since that there is hardly any utilization of scrub biomass for energy (Esteban et al., 2008). Significant amounts of shrubs as *Pterospartum tridentatum* can usually be found in the understory of *Arbutus unedo*, *Pinus pinaster* and *Eucalyptus* forests (Rego et al., 2004) and in mountain zones and in marginal land in which cultivation has been abandoned. The application of these residues in bioprocesses is favorable because the environmental problem of their disposal may be resolved, since they must be harvested in order to keep the forests clean and less vulnerable to fires.

Structural features of lignocellulosic biomass and the presence of lignin are known to inhibit biomass hydrolysis, so pretreatments are necessary to enhance biomass digestibility by altering biomass structural features (Kim and Holtzapple, 2006; Chang and Holtzapple, 2000; Lynd, 1996). However, the effect of pretreatment is strongly dependent on both the biomass composition and the operating conditions of the process, that have to be highly severe to make cellulose more accessible to the enzymes that convert the carbohydrate polymers into fermentable sugars (Mosier et al.,

* Corresponding author at: Research Unit of Textile and Paper Materials, University of Beira Interior, Av. Marquês D' Ávila e Bolama, 6201-001 Covilhã, Portugal. Tel.: +351 275 319 827; fax: +351 275 319 730.
E-mail address: fdomingues@ubi.pt (F.C. Domingues).

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2005; Galbe and Zacchi, 2007). The ideal pretreatment also should hydrolyze the hemicelluloses to its monomer sugars without producing hydrolysis inhibitors or fermentation inhibitors (Galbe and Zacchi, 2007), namely phenolic compounds, organic acids or furfural and 5-hydroxymetylfurfural, which could influence subsequent process steps and limit the ethanol production.

Pretreatment can be carried out in different ways, such as physical treatment, chemical treatment with acid or basic catalysts, and biological treatments (Esteghlalian et al., 1997). Every pretreatment process has its advantages and disadvantages and research is needed to optimize them (Hendriks and Zeeman, 2009). Among pretreatment methods, dilute acid pretreatment has been widely studied because it is effective and inexpensive. Moreover, it has been successfully applied to pretreatment of lignocellulosic materials (Sun and Cheng, 2005).

Sugars removal efficiency during pretreatment is affected by a large number of factors. In order to optimize the process, instead of performing the traditional one-factor-at-a-time approach, a response surface methodology (RSM) can be used as an optimization strategy. This approach enables the evaluation of several variables at the same time and their interactions with the response variables. In addition, to analyze the effects of independent variables, this experimental methodology generates a mathematical model that accurately describes the overall process (Myers and Montgomery, 2002). Response surface methodology has been successfully used to model and optimize different processes in ethanol production, namely pretreatment (Soderstrom et al., 2003; Canettieri et al., 2007; Rahman et al., 2007), enzymatic hydrolysis (Ferreira et al., 2009; Zhou et al., 2008; Kim et al., 2008; Lu et al., 2007), fermentation (Kim et al., 2008) or simultaneous saccharification and fermentation (Hari Krishna and Chowdary, 2000).

The aim of this study is to investigate the influence of pretreatment conditions on removal of sugars from the shrub *P. tridentatum*, and on cellulose conversion during the enzymatic hydrolysis step. Furthermore, fermentation of the filtrates was used to evaluate the influence of pretreatment on the formation of inhibitors and on its fermentabillity by *Pichia stipitis*.

2. Methods

2.1. Feedstock

P. tridentatum was used as substrate in this work. This forestry residue was harvested by "Associação de Produtores Florestais do Paúl". The shrub consisted of stalks and leaves, which were milled in a laboratory cutting mill (Retsch Mühle, Haan, Germany) and screened in a mechanical sieve shaker (Test Sieve – 5657 HAAN, Haan, Germany) following the Standard Biomass Analytical Procedures from the National Renewable Energy Laboratory (NREL, 2008). Feedstock for the pretreatment was milled to a particle size between 0.180 mm and 0.500 mm and then subjected to pretreatment with diluted acid, under different conditions.

2.2. Microorganism

P. stipitis strain CBS5773 kindly provided by INETI (Lisbon, Portugal), was used in this work (Marques et al., 2008).

2.3. Methods

2.3.1. Analysis of organic matter composition of P. tridentatum

The composition of the biomass with respect to extractives, acid-insoluble lignin, acid soluble lignin and ashes was determined following the Standard Biomass Analytical Procedures from the National Renewable Energy Laboratory (NREL, 2008).

2.3.2. Dilute acid pretreatment

Dilute acid pretreatment was carried out in 200 mL steel reactors immersed in a heating poly(ethylene)glycol (PEG) 400 bath with agitation and controlled temperature systems. Whole plant samples were treated with diluted sulfuric acid under different reaction conditions according to the central composite rotatable design (CCDR) generated using Design Expert® software version 7.1.5, from Stat-Ease, Inc., Minneapolis, USA. After pretreatment, the solid fraction was separated from the aqueous fraction by filtration. Before analysis of total reducing sugar and inhibitors concentration, the filtrates were neutralized with $Ba(OH)_2 \cdot 8H2O$ to pH 6.8 ± 0.3. The produced solid precipitate was removed by centrifugation at 4500 rpm for 5 min.

2.3.3. Central composite rotatable design

Response surface methodology (RSM) is a collection of statistical and mathematical techniques useful for developing, improving, and optimizing processes (Myers and Montgomery, 2002). The central composite design (CCD) is the most popular class of designs used to fit a second-order model. Generally, the CCD consists of a 2^k factorial with n_F, runs, 2^k axial or star runs, and n_C center runs (Montgomery, 2001). Since many variables may potentially affect the efficiency of the pretreatment process, a small type central composite rotatable design (CCDR) was employed in this study to determine the effects of independent variables on the response and the factor interactions. A total of 36 runs with different combinations of the variables were used (Table 1) to optimize the pretreatment of the forestry residue *P. tridentatum*.

Four independent variables (Table 1) were studied at five levels with four repetitions at the central point and two replicates at axial and factorial points. For each of the four variables studied, high (coded +1) and low (coded −1) set points were selected according to the results obtained with preliminary tests, taken into consideration the required experimental conditions and the results available in literature. The results of each CCRD were analyzed using Design Expert® software version 7.1.5. Both linear and quadratic effects of the four variables under study were calculated, as well as their possible interactions, on the released mass of reducing sugars by dry biomass. Their significance was evaluated by variance analysis (ANOVA). Three-dimensional surface plots were drawn to illustrate the effects of the independent variables on the dependent variable, been described by a quadratic polynomial equation and fitted to the experimental data. The fit of the models was evaluated by the determination of R^2 coefficient and adjusted R^2 coefficient.

2.3.4. Enzymatic hydrolysis

Enzymatic hydrolysis experiments on solid fraction of diluted acid pretreated *P. tridentatum* were performed at 5% (w/v) solid concentration (with basis in dry weight) in 50 mM citrate buffer pH 4.8 with poly(ethylene)glycol 4000 at a concentration of 0.25 g/g dry biomass. The reaction mixture was incubated at 50 °C for 168 h in an orbital shaker with agitation at 150 rpm.

Table 1
Process variables used in central composite rotatable design presented as coded levels.

Coded levels	X_1 – Time (min)	X_2 – Temperature (°C)	X_3 – Acid concentration (% w/w)	X_4 – Biomass/liquid ratio (g/10 mL)
−2	30.0	80	0.50	1.50
−1	48.2	100	1.41	1.80
0	75.0	130	2.75	2.25
+1	101.8	160	4.09	2.70
+2	120.0	180	5.00	3.00