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Comparative study on hydrolysis and bioethanol production from cardoon and rockrose pretreated by dilute acid hydrolysis

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ABSTRACT

Bioethanol production potential from cardoon (*Cynara cardunculus*) and rockrose (*Cistus ladanifer*), pretreated by diluted acid hydrolysis (DAH), was studied and compared by both, separate enzymatic hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) processes. DAH pretreatment allowed an increase of 5 fold to cardoon and 16 fold to rockrose in cellulose accessibility towards enzymatic hydrolysis. Both plants showed better overall fermentation efficiency and ethanol yield in SSF than in SHF also with shorter processing periods, 24 h against 72 + 24 h, respectively. Results obtained for rockrose are about half the concentration and volumetric productivity of ethanol and 60% of the overall fermentation efficiency of those obtained for cardoon. The recalcitrance of rockrose to enzymatic hydrolysis was explained by higher abundance of lignin and extractives than in cardoon. Comparing the steam explosion (SE) and DAH pretreatments, the former was more advantageous for the recalcitrant rockrose in terms of enzymatic saccharification yield.

1. Introduction

An economy based on fossil oil resources providing energy and chemicals for industrial activities needs to change gradually to meet environmental and societal issues and to allow planet sustainability. The circular economy concept started to drive European Union directives in order to decrease both dependences on fossil resources and global CO_2 emissions (van Maris et al., 2006). In this context, the production of bioethanol from renewable resources fits well to the aforementioned strategies.

Bioethanol traditionally produced from food feedstocks, called from 1st generation, is now required to change for advanced production using feedstocks not included in the food chains such as forest, agriculture or industrial residues being classified as 2nd generation bioethanol. Intensive study towards different renewable raw materials and developing new and comprehensive processes for biofuels production and application in the transportation sector is the common trend in the area. The Directive 2009/28/EC (European Parliament, 2009) for transportation fuel as a blend with at least 10% of renewable biofuel, until 2020, was revised due to such reduced production. Now the goal is 7% of biofuel blended with the indication of 0.5% from 2nd generation. Bioethanol production is increasing for being blended with commercial gasoline (Sebastião et al., 2016).

Lignocellulosic biomass (LCB), comprising about 50% of the world biomass (Fernandes et al., 2015), must be considered in order to find out new efficient biofuel production processes not competing to food raw materials. Basically, LCB polysaccharides can be hydrolysed via chemical or enzymatic routes into monosaccharides in order to be converted to biofuels or chemicals. For this purpose, due to the recalcitrance of polysaccharides in the LCB matrix, biomass pre-treatments have to be performed to attain biomass deconstruction (Moodley and Kana, 2017; Qing et al., 2017). Polysaccharides accessibility is improved and enzymatic saccharification of cellulose and hemicelluloses provides monosaccharides the right substrate for microbial fermentation to bioethanol. Among pretreatment methods, those based on physical, physicochemical, biological and chemical approaches are

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normally applied, depending on the raw materials used and the desired effect (Haghighi Mood et al., 2013).

Diluted acid hydrolysis (DAH) is the commonly used chemical pretreatment method by application of mineral acids of low concentration (normally ca. 1% concentration) (Taherzadeh and Karimi, 2007) Usually, low (≤150 °C) and high (> 150 °C) temperature DAH are distinguished. Low-temperature DAH is invoked to eliminate easily hydrolysable hemicelluloses and to increase the content and the access of cellulose in plant materials, whereas the high-temperature DAH is used to exhaustive hydrolysis of all polysaccharides.

Availability of different residual biomasses including plants growing in marginal areas without irrigation which has to be buffed preventing fire hazard could be a good feedstock choice for 2nd generation bioethanol production. *Cynara cardunculus* (cardoon) and *Cistus ladanifer* (rockrose) are examples of such plants, which can be used entirely or just part of them for this purpose (Fernandes et al., 2015; Ferro et al., 2015; Pesce et al., 2017; Rettenmaier et al., 2015). Cardoon and rockrose are perennial plants well adapted to the Mediterranean climate with low requirements of irrigation and fertilizers for cultivation between other crops (Bell and Altland, 2010; Fernández et al., 2006). In many regions, these are considered as invasive low economic value plants and make forest tracts vulnerable to the outbreak emergence and spread of fire (Nunes et al., 2005).

In this work dilute acid hydrolysis was employed as a pretreatment stage to two weedy plants growing on in the Alentejo Region of Portugal, cardoon and rockrose, followed by their enzymatic saccharification and ethanolic fermentation with *Saccharomyces cerevisiae*, to evaluate and compare their potential for bioethanol production. The efficiency of ethanol production in separate hydrolysis and fermentation (SHF) and a simultaneous saccharification and fermentation (SSF) processes for both pretreated biomasses was compared.

2. Materials and methods

2.1. Raw material

Cardoon growing rainfed was collected already dried in the field, from a farm of the Agrarian School of Beja in Portugal in July 2008. Rockrose with more than 10 years old was collected from Monte do Vento in the county of Mértola, Portugal in 2011. After collection, cardoon (stalk and leaves) and rockrose (stalks and branches with leaves) were chipped in approximately 3 cm sized pieces followed by air-drying to 8–9% humidity. Before analysis and pretreatment by dilute acid hydrolysis, cardoon and rockrose were size reduced with a knife mill and sieved in order to obtain a fraction between 40 and 60 mesh.

2.2. Dilute acid hydrolysis

Dilute acid hydrolysis was performed in agreement with a previously published work (Fernandes et al., 2016). Biomass was mixed with 6.7% (w/w) sulphuric acid solution with a hydromodulus of 10 in 500-mL universal flasks, closed with PBT screw cap with Teflon seal, at 130 °C in an autoclave for 55 min. After the reaction time, the autoclave was cooled down and the liquid fraction and solid phase were separated by vacuum filtration. The hydrolysates were filtered and analysed as described in Section 2.5. Solid fractions were thoroughly washed with distilled water until obtaining pH near 5. HPLC analysis of the last washing water did not reveal the presence or sugars (xylose and glucose) or furanic compounds (5-hydroxymethyl-furfural, furfural). The dilute acid pretreated cardoon (CDAH) and dilute acid pretreated rockrose (RDAH), were dried at 40 °C for the composition analysis (xylan, arabinan, glucan, Klason lignin) or frozen for further saccharification and fermentation studies.

2.3. Enzymatic hydrolysis

Enzymatic saccharification was carried out with 5% (w/v) of dry biomass in a final volume of 5.0 mL with 50 mM citrate buffer pH 4.8 in screw-capped 50 mL Nalgene polycarbonate centrifuge tubes (Thermo Scientific, Pittsburgh, PA). Celluclast 1.5 L and Novozyme 188 (66 FPU mL^{-1}, 197 β-glucosidase activity (CBU) mL^{-1}, respectively), commercial enzymatic complexes, from Sigma-Aldrich, were added to achieve 15 FPU and 15 CBU per gram of dry biomass, respectively. Enzymatic activities were measured according to Ghose (1987). Sodium azide, with a final concentration of 0.1%, was also used as a preservative. Assays were performed at 50 °C and 150 rpm during 72 h and after that, samples were boiled for 5 min and then centrifuged at 13000*g*. The supernatants were filtered (45 μm filter) and analysed by HPLC. All assays and the respective substrate and enzyme controls were performed in duplicate.

2.4. Fermentation studies

2.4.1. Separate hydrolysis and fermentation (SHF)

Dilute acid pretreated plants were mixed with 50 mM citrate buffer to attain 8% (w/v) on a dry weight basis of solid loading, the pH was adjusted to 4.8 and the mixtures were sterilised in the autoclave during 15 min at 121 °C. Filter sterilised Celluclast 1.5L and Novozyme 188 with 43 FPU and 50 CBU per g of dry biomass, respectively were added aseptically. Saccharification assays were performed with a total volume of 50 mL in 250 mL Erlenmeyer flasks capped with a bubble trap, at 150 rpm, 50 °C for 72 h. All assays were made in duplicate.

Active cultures of *Saccharomyces cerevisiae* NCYC 1119, from the United Kingdom National Collection of Yeast Cultures, were grown in YPD medium containing 10 gL^{-1} of yeast extract, 20 gL^{-1} of peptone and 50 gL^{-1} of glucose, at 30 °C, 130 rpm for 16 h. All fermentations were prepared using active cultures incubated at 30 °C and 130 rpm.

Fermentation after enzymatic hydrolysis (SHF) was carried out by adding sterile peptone and yeast extract to each flask, after saccharification step, in order to obtain a final concentration similar to that of YPD medium. Inoculum (5.0 mL with 0.165 g of dry yeast) was also added to each flask and fermentations were performed under semi-anaerobic conditions. Samples were taken under sterile conditions over time, centrifuged at 13000*g* at 5 °C for 10 min and the supernatant filtered with 0.22 μm for HPLC analysis.

2.4.2. Simultaneous saccharification and fermentation (SSF)

A thermotolerant yeast, *S. cerevisiae* PYCC 2613 obtained from the Portuguese Yeast Culture Collection was used in SSF experiments. Active cultures of *S. cerevisiae* were prepared in a similar way as indicated before, except at the temperature that was always 42 °C. Fermentations and saccharification were also carried out at 42 °C in a similar way as in SHF, with 5.0 mL of *S. cerevisiae* inoculum (0.017 g dry weight). All fermentation assays were done in duplicate.

For caring out SSF assays preparation was made in the same conditions as the previous SHF experiments but mixing solid materials, citrate buffer, supplements, enzymes and inoculum in the beginning of the experiments.

2.5. Chemical analysis

2.5.1. Biomass and hydrolysates

Biomass raw materials (cardoon and rockrose), dilute acid hydrolysis residues (CDAH, RDAH) and respective liquid fractions were characterized according to Laboratory Analytical Procedures (LAP) from the National Renewable Energy Laboratory (NREL) (Sluiter et al., 2008a,b,c). A Soxhlet' s extraction with acetone was carried out for 6 h in order to isolate the extractives in acetone and to prepare acetone extractive-free samples for further analysis. Water-soluble extractives were determined in acetone extractives-free plant materials by reflux