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Enzymatic saccharification and bioethanol production from *Cynara cardunculus* pretreated by steam explosion

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HIGHLIGHTS

- Steam explosion of cardoon (CSE) enhances enzymatic accessibility of cellulose.
- CSE promotes disruption of interfibrillar surfaces and partial degradation of lignin.
- Alkali extraction of CSE (CSEOH) increases lignin removal and saccharification.
- CSEOH simultaneous saccharification and fermentation (SSF) produced 20 g L^{-1} ethanol.
- Bioethanol by SSF was more efficient than separate hydrolyses and fermentation (SHF).

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ABSTRACT

The correct choice of the specific lignocellulosic biomass pretreatment allows obtaining high biomass conversions for biorefinery implementations and cellulosic bioethanol production from renewable resources. *Cynara cardunculus* (cardoon) pretreated by steam explosion (SE) was involved in second-generation bioethanol production using separate hydrolysis and fermentation (SHF) or simultaneous saccharification and fermentation (SSF) processes. Steam explosion pretreatment led to partial solubilisation of hemicelluloses and increased the accessibility of residual polysaccharides towards enzymatic hydrolysis revealing 64% of sugars yield against 11% from untreated plant material. Alkaline extraction after SE pretreatment of cardoon (CSEOH) promoted partial removal of degraded lignin, tannins, extractives and hemicelluloses thus allowing to double glucose concentration upon saccharification step. Bioethanol fermentation in SSF mode was faster than SHF process providing the best results: ethanol concentration 18.7 g L^{-1}, fermentation efficiency of 66.6% and a yield of 26.6 g ethanol/100 g CSEOH or 10.1 g ethanol/100 g untreated cardoon.

1. Introduction

Lignocellulosic biomass (LCB) is the most abundant renewable resource on Earth, comprising about 50% of world biomass. LCB is outside the human food chain and its energetic content exceeds by many times the world basic energy requirements (Fernandes et al., 2012). These features make it an important option as feedstock, as a relatively inexpensive raw material, for second generation bioethanol production.

Cynara cardunculus (cardoon) is a perennial plant well adapted to Mediterranean climate with low requirements of irrigation and fertilizers for cultivation than other crops (Fernández et al., 2006). It has been largely investigated as fuel crop (Gominho et al., 2011; Lag et al., 2009; Mantineo et al., 2009; Oliveira et al., 2012). Besides the traditional use of the blanched stalks as an edible vegetable and the use of the flowers as a rennet substitute to make cheese (Fernández et al., 2006; Ramos et al., 2014) the plant has other applications. The capitula includes seeds which are used for oil production that can be used for biodiesel production (Fernández et al., 2006), hairs and pappi, used for paper production (Gominho et al., 2009). Stalks can be used for paper

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production (Gominho and Pereira, 2006) or biogas (Oliveira et al., 2012) and other uses (Mancera et al., 2008).

Cardoon is well adapted to the edaphoclimatic conditions of Alentejo, South region of Portugal, where it could be exploited as feedstock according to the biorefinery concept. Cardoon extractives fraction is a rich source of bioactive compounds with direct application in food and pharmaceutical industries (Ramos et al., 2014; Velez et al., 2012). Cardoon polysaccharides can be converted to monosaccharides for bioethanol production and for many other applications.

Three major structural groups compose LCB: cellulose, hemicelluloses and lignin. It also includes relatively low amounts of extractives, tannins, ashes, proteins and other compounds, which are not responsible for the complex macromolecular structure of LCB (Balat, 2011). Due to the recalcitrance of the LCB matrix composed by the major groups stated above, enzymatic hydrolysis of cellulose is rather difficult compared to the breakdown of other renewable materials such as starch. Thus, pretreatment process in which the cellulose is made accessible for further conversion has to be applied on LCB before the enzymatic hydrolysis step. The choice of pretreatment depends largely on the purpose of the pretreatment of biomass, its economic assessment and the environmental impact. Only a small number of pretreatment methods were reported as being potentially cost-effective thus far. These include steam explosion, liquid hot water, concentrated acid hydrolysis and dilute acid pretreatments (Balat, 2011). However, it is not possible to define the best pretreatment method as it depends on many factors, such as the type of lignocellulosic biomass and the specific desired products. In fact, steam explosion (Martinez et al., 1990) and dilute acid hydrolysis (Ballesteros et al., 2007; Shatalov and Pereira, 2014, 2011) were already used as pretreatment stages before the enzymatic saccharification of cardoon. Bioethanol concentration achieved with loading of 10% solids in simultaneous saccharification and fermentation (SSF) mode was 18.1 and 23 g L^{-1} applying the steam explosion (Oliva et al., 2008) and the dilute acid hydrolysis (Ballesteros et al., 2008) pretreatments, respectively.

Recently large scale industrial plants, with a total annual capacity around 430 million L of bioethanol are being widely installed in United States, Europe and also Asia, showing that if crop residues are properly managed and harvested, they are sustainable. Crop residues as a rentable raw material is very important since after bioethanol production other high value added products will be rising in the same industrial plants providing the development of real biorefineries from new residues platforms. The success of an initial industry based on residues will enable a much larger industry based on perennial grasses and forest products, both of which can significantly enhance the environmental performance of cellulosic biofuels (Dale and State, 2015).

The objective of this work was to study steam explosion pretreatment of cardoon biomass in order to enhance cellulose saccharification and to increase bioethanol fermentation. Accordingly, different chemical and structural analysis were done in order to compare both cardoon biomass with and without pretreatment and bioethanol production by separate hydrolysis and fermentation (SHF) or by simultaneous saccharification and fermentation (SSF).

2. Methods

2.1. Raw material

Cardoon growing rainfed older than 3 years was collected already dried in the field, from a farm of the Agrarian School of Beja in July 2008. Cardoon (stalk and leaves) was size reduced and sieved to obtain particles between 40–60 mesh for the chemical characterisation. For steam explosion treatment (SE) cardoon was reduced to 18 mesh.

2.2. Biomass treatments

2.2.1. Steam explosion pretreatment

Steam explosion treatment of cardoon was carried out in a laboratory pilot unit designed by Latvian company "FIL & KO" for the Latvian State Institute of Wood Chemistry. Cardoon was treated by saturated steam for 1 min at 235 °C, 3.2 MPa in a 0.5 L reactor and a receiving chamber of 30 L. The reactor was equipped with a quick-opening ball valve and an electronic device programmed for accurate control of steam time and temperature. The severity factor (log R_0) for steam explosion treatment was 3.97 calculated using the reaction ordinate R_0, which can be expressed as:

$$R_0 = t \times \exp^{[(T-100)/14.75]} \quad (1)$$

where duration of treatment (t, min) and temperature (T, °C) express the severity against the base temperature, T, using the reference of 100 °C.

Resultant water insoluble solid fraction hereinafter CSE was air dried. The samples used for the enzymatic assays were hydrated during 24 h with deionized water, vacuum filtered and then thoroughly washed, to remove inhibitors impregnated in the material.

2.2.2. Alkaline extraction

Samples pretreated by steam explosion, CSE, were soaked for 24 h in distilled water, vacuum filtered and then treated for 15 min with 2% sodium hydroxide solution at hydromodulus 5 according to (Fernández-Bolaños et al., 2001). Afterwards, the obtained residue, hereinafter CSEOH, was thoroughly washed with hot deionized water till alkali removal (pH of filtrate ca 6.8), dried at 40 °C or frozen for further analysis or enzymatic hydrolysis, respectively.

2.3. Enzymatic hydrolysis

Saccharification was carried out with 5% of dry biomass in a final volume of 5 mL with 50 mM citrate buffer pH 4.8 in screw-capped centrifuge tubes. Celluclast 1.5 L and Novozyme 188, commercial enzymatic complexes, from Sigma Aldrich, were added to achieve 15 FPU and 15-β-glucosidase IU (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 centrifuged at 13,000 g. The supernatants were analysed by HPLC, after filtration through 0.45 μm filter. All assays, respective substrate and enzyme controls were performed in duplicate.

2.4. Microorganisms, growth conditions and fermentation

Two *Saccharomyces cerevisiae* strains were used, namely NCYC 1119 for SHF experiments and the thermotolerant strain PYCC 2613 for SSF assays. Active cultures were prepared in 250 mL Erlenmeyer flasks with 100 mL of YPD growth medium containing 10 g L^{-1} of yeast extract, 20 g L^{-1} of peptone and 50 g L^{-1} of glucose. The flasks were incubated during 16 h at 130 rpm and 30 °C (NCYC 1119) or 42 °C (PYCC 2613), respectively.

SHF and SSF studies were performed using 43 FPU of Celluclast 1.5 L and 50 CBU of Novozyme 188/g of dry biomass with 8% of solids. All assays were done with total volume of 50 mL in closed 250 mL Erlenmeyer flasks.