Renewable Energy xxx (2015) 1–5

Contents lists available at ScienceDirect

Renewable Energy

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

Review

Ethanol production from halophyte *Juncus maritimus* using freezing and thawing biomass pretreatment

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ARTICLE INFO

Article history:
Received 24 October 2014
Received in revised form
5 April 2015
Accepted 5 July 2015
Available online xxx

Keywords:
Juncus maritimus
Dilute acid pretreatment
Freezing/thawing pretreatment
Enzymatic saccarification
Alcoholic fermentation

ABSTRACT

Juncus maritimus contains (41.5 ± 0.3)% cellulose and (31.34 ± 0.2)% hemicellulose on dry solid (DS) basis and has the potential to serve as a low cost feedstock for ethanol production. Dilute acid or freezing/thawing pretreatments and enzymatic saccharification were evaluated for conversion of halophyte plant from *J. maritimus* cellulose and hemicelluloses to monomeric sugars. The maximum concentration of released glucose from *J. maritimus* (53.78 ± 3.24) g L^{-1}) by Freezing/thawing pretreatment and enzymatic saccharification (55 °C, pH 5.0 and 48 h) using CellicCTec2 from Novozymes and (49.14 ± 5.24) g L^{-1} obtained by dilute acid pretreatment. The maximum yield of ethanol from acid pretreated enzyme saccharified *J. maritimus* hydrolyzate by *Saccharomyces cerevisiae* strain was (84.28 ± 5.11)% of the theoretical yield with a productivity of (0.88 ± 0.16)g L^{-1} h^{-1}. It was (90.87 ± 1.94)% of the theoretical yield with a productivity of (1.04 ± 0.10) g $L^{-1}h^{-1}$ for freezing/thawing pretreated plant and enzymatic hydrolysis by CellicCTec2.

1. Introduction

Renewable energies are gaining more interest as fossil energy becomes more scarce and expensive. In fact, the exploitation of different biomass sources may compensate a major part of future energy demands. In this context, lignocellulosic biomass represents a potential candidate for bioethanol production due to its availability and environmental benefits [1].

Bioethanol is mainly produced from food sources such as sugarcane, rice, cassava, and corn. Consequently, the exploitation of a large amount of food sources for bioethanol production would increase food cost [2]. Therefore, researchers are focusing on finding renewable resources other than human food resources. In this context, lignocellulosic materials such as agricultural residues, forestry wastes and municipal materials constitute a potential source for bioenergy production [3]. Lignocellulosic biomass mainly consists of three polymers which are cellulose, hemicelluloses and lignin [4]. Among these components, carbohydrates (cellulose and hemicelluloses) can be saccharified and eventually fermented in order to obtain bioethanol [5].

The pretreatment process is the most important step in cellulose-to-ethanol technology, since it can solubilize hemicelluloses, reduce cellulose's crystallinity and increase the materials' porosity [4]. There is a number of available pretreatment technologies including physical, biological, physical-chemical (liquid hot water, steam explosion ammonia fiber explosion, Instant Controlled Pressure Drop [6,7]) and chemical (acid, alkaline, wet oxidation, ozonolysis) [8]. However, the dilute acid is the most widely used method for lignocellulosic material pre-treatment due to its relatively low cost, ease of use [9] and high efficiency [10]. It removes and hydrolyzes up to 90% of hemicelluloses making the cellulose fraction more accessible to enzymatic hydrolysis. While, this pretreatment presents important drawbacks related to the formation of toxic compounds (furfural and hydroxymethyl furfural), issued from sugar degradation, as inhibitors for the fermentation step. Also, the mineral acids are equipment stainless-corrosive. In addition, the environmental problems caused by the waste streams liberated from the dilute acid pretreatment process [10]. Its mechanism comprises series of reactions. First, the protons are diffused through the wet lignocellulosic matrix followed by the protonation of the ether-oxygen link between the monomeric

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http://dx.doi.org/10.1016/j.renene.2015.07.010
0960-1481/

sugars. Then, the rupture of the ether bond was achieved for generation of a carbocation. Finally, the latter undergoes a solvation with water in order to generate sugars monomers, oligomers and polymers depending on the ether connection that is broken with regeneration of proton [11].

Glycophytes represent a large proportion of plant species, which are sensitive to high soil salinity in contrast to halophytes that naturally tolerate such conditions. In fact, halophytes can be found in alkaline semi-deserts, salt marshes, steppes, and sea coasts. The use of halophytes plants as a bioenergy biomass is beneficial since they don't compete with conventional crops for high quality soil and may be irrigated with saline water [12,13]. Consequently, there is a necessity for exploiting saline lands in producing non-foodligno-cellulosic biomass, which may be later transformed into ethanol without compromising human food production. Hereby, halophytes, which produce plenty of biomass in saline environment, constitute a potential candidate for bioenergy production.

In this work, our main objective was to produce ethanol from halophyte biomass using *Juncus maritimus* as a raw material for bioenergy production. In order to achieve this objective, three steps processes were performed. First, the plant was treated by two various methods, which were dilute acid and freezing/thawing. To our knowledge, the last method has never been used for biomass pretreatment in order to produce ethanol. However, it is used as a conservation process (cryoconservation) in several domains such as food industry [14], medical technology, pharmaceutical industry (i.e. to store drug substances and protein for a long periods) [15], biochemistry and microbiology (i.e. to conserve proteins, tissues and cells) [16]. Several studies indicate that cells' membrane systems are the primary site of freezing injuries in plants [17,18]. In fact, under a temperature around −20 °C (frozen state), the plasma membrane of plant cells undergoes a rupture by the formation of a large amount of intracellular ice. When cells are exposed to high temperature (thawed state), the intracellular frozen cells are subjected to further ultra structural changes during the thawing process resulting in serious cell damage [19].

Thus, the freezing/thawing method could be used to breakdown cell walls to enhance the accessibility of their polysaccharides to the hydrolyzing enzymes.

Then, an enzymatic saccharification was performed in order to produce fermentable sugar. Finally, the latter was converted into bioethanol using *S. cerevisiae* yeast.

2. Materials and methods

2.1. Materials

J. maritimus Lamk. is a salt marsh plant. Plant-shaped tufts grow up to 1 m height. The rhizoma generates long and parallel stems that are all radical, naked, and hard spines at the end.

5-year old *J. maritimus* was harvested in its native salty ecosystem from the Tunisian region Soliman sabkha (N 36 42 14, E 10 27 21). This region is located on the Mediterranean coast 40 km of Tunisian and characterized by upper semi aridbioclimatic. Average of annual rainfall estimated about 450 mm.

Aerial parts were collected in March 2013 corresponding to the flowering period. It was dried at 50 °C for 48 h and milled in a hammer mill to attain a particle size of 3 mm. The milled *J. maritimus* was then stored at room temperature.

CellicCTec2 (0,43 pNPG U ml^{-1}, 61, 25 CMC U ml^{-1}) and CellicHTec2 (0,25 pNPG U ml^{-1}, 92, 3 CMC U ml^{-1}) enzymes from Novozymes (Denmark) and Accelerase 1500 (0,25 pNPG U ml^{-1},38,3 CMC U ml^{-1}) from Genoncor were used for the enzymatic hydrolysis of *J. maritimus*. There are states of the art on enzymes that prove effective on a wide variety of pretreated lignocellulosic materials for the conversion of the carbohydrates in these materials to simple sugars prior the fermentation.

The endoglucanase activity is standardized on the basis of its activity on carboxymethylcellulose (CMC). A CMC activity unit liberates 1 μmol of reducing sugars in 1 min under specific assay conditions of 50 °C and pH 5.

The β-glucosidase activity is standardized on the basis of activity on pNPglucoside. One pNPG unit denotes 1 μmole of Nitrophenol liberated from para-nitrophenyl-β-D-glucopyranoside per minute at of 55 °C and pH 5.

A strain of yeast *S. cerevisiae* was selected as a model strain for fermentation of simple sugars. The fermentation was achieved using fresh commercial baker's yeast *S. cerevisiae* (Tunisian Society of yeasts, *S. cerevisiae* purchased from local market) [20].

2.2. Dilute acid pretreatment of J. maritimus

Milled *J. maritimus* was slurries in1% H_2SO_4 (5% on dry solid basis, DM, w/v) and pretreated in an autoclave at 121 °C for1 h. The wet material was filtered. The solid residue was washed 3 to 4 times by distilled water to neutralize pH. It was dried at 50 °C for 48 h [21].

2.3. Freezing/thawing pretreatment

5 g of milled *J. maritimus* were frozen in a conventional freezer (Classical Freezer, LG, GN-392, Tunisia) for 24 h at −20 °C [22] and immediately thawing in water bath at 100 °C for 15 min just for obtaining a thawed material. The sample was then filtered and dried at 50 °C for 48 h. Finally, the residue was reserved for the enzymatic hydrolysis step.

2.4. Enzymatic saccharification

The enzymatic saccharification of the diluted acid and the freezing/thawing pretreated *J. maritimus* was performed for 48 h at 55 °C and pH5 adjusted with 5 mM sodium acetate buffer and adding enzymes at each enzyme dose of 1 mg g^{-1} DS (dry substrate) of *J. maritimus* [23]. Samples (1 mL) were kept at 20 °C before HPLC analysis. These experiments were replicated three times.

2.5. Yeast cultivation

S. cerevisiae was used as an ethanol fermentation strain. The yeast strain was maintained on agar plates made from 5 g L^{-1} yeast extract, 5 g L^{-1} peptone, 20 g L^{-1} d-glucose, and 20 g L^{-1} agar. Inoculation flasks were prepared by autoclaving 100 mL of 50 g L^{-1} glucose, 1 g L^{-1} KH_2PO_4, 1 g L^{-1} $MgSO_4 + 7H_2O$, 5 g L^{-1} peptone and 5 g L^{-1} yeast extract. The medium was incubated for 24 h at 30 °C with shaking (150 rpm) prior to use.

2.6. Fermentation

The batch fermentation experiments were carried out in 100 mL flasks under anaerobic conditions with working volumes of 20 mL.

The hydrolysates (15 mL) of the solid fraction resulting of *J. maritimus* pretreatment were used as substrates with 2 mL of YPX10 (200 g L^{-1} yeast extract and 400 g L^{-1} peptone)and 2 mL of the yeast suspension. Fermentation was incubated for 24 h at 37 °C with shaking (100 rpm) [24]. These experiments were replicated three times.

For calculation of ethanol yield the following equation was applied [25,26].

Please cite this article in press as: N. Smichi, et al., Ethanol production from halophyte *Juncus maritimus* using freezing and thawing biomass pretreatment, Renewable Energy (2015), http://dx.doi.org/10.1016/j.renene.2015.07.010