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Pretreatment and enzymatic saccharification of new phytoresource for bioethanol production from halophyte species

Neila Smichi [a,*], Yosra Messaoudi [a], Riadh Ksouri [b], Chedly Abdelly [b], Mohamed Gargouri [a]

[a] Biocatalysis and Industrial Enzymes Group, Laboratory of Microbial, Ecology and Technology, Carthage University, National Institute of Applied Sciences and Technology, BP 676, 1080 Tunis Cedex, Tunisia
[b] Laboratory of Extremophile Plants, Center of Biotechnology of Borj Cedria, BP 901, 2050 Hammemlif, Tunisia

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ABSTRACT

In this work, new halophyte plant biomass is proposed as raw material for bioenergy production. The bioconversion process of lignocellulosic materials into ethanol needs a pretreatment before enzymatic hydrolysis of vegetal material to increase the yield of fermentable sugars. Various mechanical and chemical pre-treatment processes were investigated in an attempt to facilitate the solubilization of large fraction of hemicelluloses.

The fungi *Trichoderma* spp., *Aspergillus niger*, *Penicillium italicum* and *Fusarium* spp. have been developed on the halophyte plant, *Juncus maritimes*, producing polysaccharides hydrolases activities, i.e. endoglucanasecatalyses cellulose hydrolysis, and beta-glucosidasecatalyses hydrolysis of terminal non-reducing residues in beta-glucosides. The saccharification was carried out with an enzymatic preparation extracted from filamentous fungus *Trichoderma* spp. Experimental Doehlert design was performed to optimize the reducing sugars concentration. High sugar yields (8.5 g/L) were obtained using a small amount of the extracted enzyme without stirring.

The highest enzymatic activities of endoglucanase and beta-glucosidases were produced by *Trichoderma* spp. at 22.12 and 0.07 U/mg, respectively.

1. Introduction

Cellulose and hemicelluloses typically comprise two-thirds of the dry mass of a plant. They can be transformed into fuel by pyrolysis. Pyrolysis is achieved at 300 °C–500 °C. This method results in the decomposition of cellulose into lighter compounds (C_2H_4, CO) which, for some part, are toxic for human and animals, thus implying evident risks [1].

Since cellulose and hemicelluloses are polysaccharides, they can be hydrolyzed by enzymatic treatment to simple sugars and possibly fermented to ethanol [2]. Nowadays, ethanol is the major biofuel in use [3]; it is capable to be used for conventional automobiles if blended at less than 10% [4]. To break the lignin seal and hemicellulose sheathing over cellulose, numerous pre-treatment methods have been suggested during the last decades [5]; they usually imply a pretreatment stage of High Pressure Steam Disintegration possibly coupled to dilute acid hydrolysis as pretreatments [1].

The High Pressure Steam Disintegration process uses high temperature (up to 200 °C) and high pressure (up to 3 MPa). It aims at increasing the internal diffusivity thus enhancing the degradation of cellulose and hemicelluloses into more fermentable compounds. However, it can lead to a great thermal degradation of sugars, thus producing some molecules (furfural, hydroxymethyl furfural), which can inhibit the ethanol production. The energy consumption of both processes of pyrolysis and High Pressure Steam Disintegration is generally very high [6].

The most widely used method for pre-treatment of lignocellulosic material is dilute acid. This pretreatment limits the thermal degradation of polysaccharides and has good results in cellulose to sugars conversion yields [7].

Bioethanol is currently produced almost exclusively from either sucrose (sugarcane or beets) or starchy feedstock (corn and cassava [4]) using *Saccharomyces yeast* [8,9]. The Chinese government started to regulate maize-based ethanol production which has left

* Corresponding author. Tel.: +216 71 703 717x6110; fax: +216 71 704 329.
E-mail addresses: neila.smichi@yahoo.fr (N. Smichi), messaoudi_yosra@yahoo.fr (Y. Messaoudi), riadh.ksouei@cbbc.rnrt.tn (R. Ksouri), abdelly@cbbc.rnrt.tn (C. Abdelly), mohamed.gargouri@insat.rnu.tn (M. Gargouri).

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sugarcane and cassava as the most important sources of bioethanol production in future [4].

However, since starchy feedstocks are primary food for humans and feed for animals, their conversion to bioethanol is not desirable. If biofuels ever become alternatives for fossil fuels, their production must be based on lignocellulolytic feedstock. Lignocellulosic biomass and wastes represent a vast potential alternative resource for ethanol production [10–12].

Plant biomass may be a raw material for the production of value added products, such as biofuels, biochemicals, biopesticides, and biopromoters. For bioethanol production from lignocellulosic material, the primary requirement is the hydrolysis of lignocelluloses into fermentable sugars by lignocellulolytic enzymes [13–16].

Scientific communities show strong interests to cellulases for their applications in various sectors such as pulp and paper industries, textile industry and starch processing.

These enzymes were also studied for their essential role during the step of saccharification in bioethanol processing [17].

Actually, the use of current commercial preparations of cellulases for bioconversion of lignocellulosic biomass is not feasible economically; this is why microbial cellulases found applications in various industries [18].

The production of cellulase is a key factor in the hydrolysis step and it is essential to make the process viable [19]. Among the cellulolytic fungi, *Trichoderma* and *Aspergillus* have been extensively studied in their cellulose enzymes degrading secretions [20].

The cellulase system consists of three classes of enzymes: cellobiohydrolases (CBH, EG 3.2.1.91) cleaving cellobiosyl units from the ends of cellulose chains, endoglucanases (EG, EC 3.2.1.4) cleaving internal glucosidic bonds, and β-glucosidase (EC 3.2.1.21) cleaving glucose units [19].

This study suggests a new lignocellulosic source to produce ethanol such as halophyte species, which are colonizing salty lands, littoral regions and sandy beaches. Doehlert experimental design was used in an attempt to improve the amount of reducing sugars in the presence of enzymatic preparation extracted from a filamentous fungi culture.

Halophyte plants are so abundant that they can be valorized in many issues such as bioethanol. Actually, in Tunisia, halophyte plants cover a surface of 537 790 ha per stepic species. This surface is almost the half of the forester surface which is about 1, 141, 628 ha in Tunisia [21].

Retama retam is used in this study as a biomass to produce bioethanol. The superficie of this halophyte plant is about 210 825 ha [21]. This work is subscribed in a long-term objective of recovering of plant biomass from saline environment by its conversion into bioethanol.

The main objective is to demonstrate the feasibility of using biomass from halophyte plants as raw material for bioethanol production and for general biorefinery by the mean of Doehlert experimental design.

2. Materials and methods

2.1. Raw material

Halophyte plants called *Retama retam* and *Juncus maritimus* were used in all the experiments. It has been collected from Soliman salt depression (Tunis). It was dried at 105 °C for 24 h. Then, it was ball-milled to obtain a powder (1000 μm).

Retama retam was selected as substrate for ethanol production and *J. maritimus* was used as carbon source for fungi culture.

2.2. Determination of the distribution of cell wall constituents (cellulose, hemicelluloses and lignin)

The determination of cell wall constituents was performed by the analytical method described by Goering and Van Soext [22].

2.3. Diluted acid pretreatment

Acid pretreatment was carried out with 2 g of *Retama retam* plant powder (dry weight base) and 40 ml of 1% sulfuric acid solution at 121 °C for 30 min. The wet material was filtered. The water – insoluble fraction was washed 2–3 times with distilled water to neutralize pH and the liquid fraction was used to determinate the concentration of reducing sugar released from hemicellulose solubilization [23].

The solid residue was washed three times by distilled water and dried at room temperature. It was used for saccharification step.

2.4. Micoorganisms and media

The strains utilized are *Aspergillus niger*, *Trichoderma* spp., *Penicillium italicum* and *Fusarium* spp. They were maintained on PDA at room temperature for 7 days for spore production.

Selected filamentous fungi were used for polysaccharides hydrolases production.

The mineral salt medium for endoglucanase, endoxylanase, β-glucosidase and β-xylosidase production was composed as: KCl(1 g/L); $MgSO_4$(0.5 g/L); KH_2PO_4(6 g/L); $NaNO_3$(4.3 g/L); $(NH_4)SO_4$(1.4 g/L); yeast extract (2 g/L). *J. maritimus* plant (10 g/L) was used as carbon source for enzyme production and 1 mL/L of oligoelement solution (composed of $MnSO_4H_2O$: 1.6 g/L; SO_4H_2O: 1.4 g/L; $FeSO_47H_2O$: 5 g/L; $CoCl_2$: 2 g/L; KH_2PO_4: 14.3 g/L). The culture medium pH was adjusted at 5.5 and it was sterilized for 20 min at 121 °C. The culture medium was incubated in a 300-rpm shaker at 25 °C for 10 days. The mycelium was then removed by filtration and centrifugation at 4000 rpm during 30 min at 4 °C.

2.5. Enzyme assays

Determination of endoglucanase and endoxylanase activities, contained in different enzyme preparations, was performed spectrophotometrically using respectively CMC and Xylan as substrates.

2.5.1. Endoglucanase assay

Carboxymethyl cellulose (CMC1%) solution was prepared in 100 mM sodium acetate buffer (pH5.0). 500 μL of CMC solution was added to500 μL of the test enzyme solution and the volume was completed to 1 mL with distilled water. The mixture was incubated for 10 min at 45 °C with DNS, and then heated at100 °C for 8 min, and 1mLof H_2O was added. The absorption was measured at 540 nm.

2.5.2. Endoxylanase assay

Xylan (1%) was prepared in sodium acetate buffer (100 mM, pH 5). A volume of 500 μL was added to the enzymatic extract and the volume was completed to 1000 μL with distilled water. After incubation for 10 min at 45 °C, 1 mL of DNS was added to the reaction medium. The mixture was heated at100 °C for 8 min, 1 mL of H_2O was then added and the absorption was measured at 540 nm.

2.5.3. β-glucosidase and β-xylosidase assay

β-glucosidase activity was measured by spectrophotometry using pNPGlu (para-nitro-phenyl-β-D-glucopyranoside) or cellobiose (β-(1,4)-glucobiose) as substrate. For β-xylosidase activity, pNPX (para-nitro-phenyl-β-D-xylopyranoside) was used as