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Liquid nitrogen pretreatment of eucalyptus sawdust and rice hull for enhanced enzymatic saccharification

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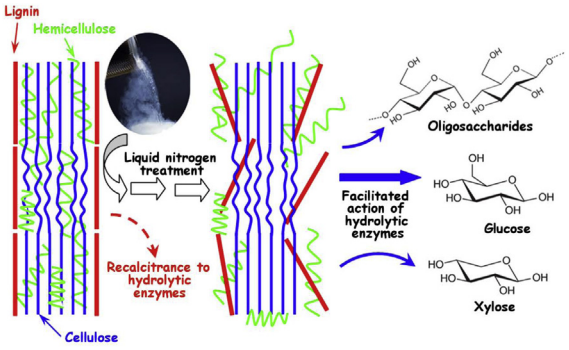
HIGHLIGHTS

- Cryogenic grinding with liquid nitrogen is a method of powdering plant biomass.
- Cryocrushing was used for the first time as pretreatment of lignocelluloses.
- Cryocrushing disorganized the fibers, without lignin destruction.
- Saccharification of eucalyptus sawdust and rice hull improved more than 10 times.
- Cryocrushing did not improve the saccharification of pure cellulose.

GRAPHICAL ABSTRACT



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ABSTRACT

In this work, liquid nitrogen was used for the first time in the pretreatment of plant biomasses for purposes of enzymatic saccharification. After treatment (cryocrushing), the initial rates of the enzymatic hydrolysis of eucalyptus sawdust and rice hull were increased more than ten-fold. Cryocrushing did not modify significantly the contents of cellulose, hemicellulose and lignin in both eucalyptus sawdust and rice hulls. However, substantial disorganization of the lignocellulosic materials in consequence of the pretreatment could be observed by electron microscopy. Cryocrushing was highly efficient in improving the saccharification of the holocellulose component of the plant biomasses (from 4.3% to 54.1% for eucalyptus sawdust and from 3.9% to 40.6% for rice hull). It is important to emphasize that it consists in a simple operation with low requirements of water and chemicals, no corrosion, no release of products such as soluble phenolics, furfural and hydroxymethylfurfural and no waste generation.

1. Introduction

Lignocellulosic biomasses are composed mainly of three polymers: cellulose, hemicellulose, which together represent up to 80% of its dry weight, and lignin. For decades, extensive research has been conducted for adding value to the plant polysaccharides cellulose and hemicellulose. Valuable fine chemicals and biofuels

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can be obtained especially after saccharification and fermentation (Maurya et al., 2015; Wang et al., 2016). As lignin comprises 15–30% of the biomass, in the last years, strategies for adding value to this material have also been proposed (Linger et al., 2014; Santos et al., 2016). Actually, over 200 value-added compounds can be derived from lignocellulosic biomasses by various treatment methods (Isikgor and Becer, 2015).

All lignocellulosic biomasses are, at least in principle, suitable for this purpose. *Eucalyptus grandis* sawdust (cellulose 54.1%; hemicellulose 18.4%; lignin 21.5%) and rice hull (cellulose 28.7–35.6%; hemicellulose 12.0–29.3%; lignin 15.4–20.0%) are wastes largely disposable in several countries and, until now, less exploited than other agricultural and forestal wastes as substrates for the production of valuable chemicals including fermentable sugars.

Enzymatic hydrolysis of holocellulose (cellulose plus hemicellulose) is generally the limiting step in the production of fine chemicals and biofuels especially for two reasons: the recalcitrance of the crystalline cellulose itself and the fact that holocellulose is closely merged and bonded chemically with lignin. The latter acts as a physical barrier against the enzymatic attack. To overcome the problems caused by biomass recalcitrance and improve the saccharification process, two types of approaches can be used. In the first one, genetic manipulations have been used with the purpose of creating transgenic plants with a more open cell wall containing less lignin and cellulose with lower crystallinity (Loqué et al., 2015; Wang et al., 2016). In the second, and until now most used, the lignocellulose biomasses are submitted to several kinds of pretreatments aiming to remove lignin and breaking down the cellulosic crystalline structure. For the latter several physical, chemical, physicochemical and biological pretreatments have been used. The long list includes milling, steam explosion, exposure to ionizing radiation, ozonolysis, treatment with acid, alkali, oxidative agents, organic solvents and ionic liquids or combinations (Karimi and Taherzadeh, 2016; Rabemanolontsoa and Saka, 2016; Zhang et al., 2016). In general, these processes require high amounts of energy, excess water use and expensive chemicals. Moreover, they can generate undesirable by-products and inhibitors which will affect enzymatic hydrolysis and fermentation (Jönsson and Martin, 2016). Biological pretreatments using white-rot fungi can be a promising technology due to several advantages such as minimal waste production, no generation of toxic compounds and low energy requirements (Sindhu et al., 2016). However, they also have drawbacks, the most important being the prolonged treatment times and the low digestibility achieved in most cases.

Cryogenic grinding (cryocrushing) is a method of powdering materials at sub-zero temperatures generally using liquid nitrogen. Cryocrushing has been employed as an alternative method for producing nanofibers in which fibers are frozen using liquid nitrogen and subsequently subjected to high shear forces (Chakraborty et al., 2005). When high impact forces are applied to the frozen fibers, ice crystals exert pressure against the cell walls, causing them to rupture and thereby to release the microfibrils (Wang and Sain, 2007). Liquid nitrogen is used for many cooling and cryogenic applications. At normal pressure, it boils at 77 K (−195.8 °C). Grinding frozen biological samples with liquid nitrogen using mortar and pestle is a widely employed method for extracting intracellular compounds. Based on these notions, the main objective of this study was to investigate the potential of using cryocrushing as a pretreatment to enhance the enzymatic hydrolysis of *Eucalyptus grandis* sawdust and rice hulls. A mathematical analysis of the hydrolysis curves was done in order to obtain quantitative parameters about the modifications caused by cryocrushing. Furthermore, in order to characterize the physico-chemical modifications, the pretreated fibers were also evaluated by Fourier transform infrared spectroscopy (FTIR), scanning electron microscopy and X-ray diffraction.

2. Material and methods

2.1. Materials

Two types of lignocellulosic biomasses were used, *Eucalyptus grandis* sawdust and rice hulls. The biomasses were air-dried and grounded in a mortar. The fractions with the particle size of 20–40 mesh were used for the experiments. The dried biomasses were stored in polyethylene plastic containers. Hydrolytic enzymes (Cellic®CTec 2 and Cellic®HTec2) were kindly supplied by Novozymes (Araucária, PR, Brazil). Enzymatic substrates carboxymethylcellulose, birchwood xylan, *o*-nitrophenyl-β-glucopyranoside, as well as microcrystalline cellulose (Avicel) and 3,5-dinitrosalicylic acid were purchased from Sigma Chemical Co. All chemicals were of analytical grade.

2.2. Cryocrushing procedure

In a typical run, 5 g of each dried biomass powder plus 50 mL of liquid nitrogen were vigorously crushed in a mortar with a pestle until complete evaporation of the latter. The materials were washed twice with distilled water, filtered and the insoluble materials dried at 40 °C in a forced convection oven until constant weight. These final preparations were used for the chemical and physical analyses of the structural components of the cell wall.

2.3. Determination of total soluble phenolics, furfural and hydroxymethylfurfural

The soluble materials from untreated and treated biomasses were analyzed for the presence of total soluble phenolics (Singleton and Rossi, 1965), and furfural and hydroxymethylfurfural (Dong et al., 2013) using conventional techniques.

2.4. Evaluation of the activities of the commercial enzyme cocktails Cellic®CTec 2 and Cellic®HTec2

Endocellulase and endoxylanase activities were assayed in 50 mmol.L^{-1} sodium acetate buffer at pH 5.0 and 50 °C using, respectively, 1% carboxymethylcellulose and xylan as substrates. The released reducing sugars were quantified by the 3,5-dinitrosalicylic acid (DNS) reagent (Miller, 1959) using glucose or xylose as standards, respectively. The β-glucosidase activity was assayed in 50 mmol.L^{-1} sodium acetate buffer at pH 5.0, at 50 °C, using the synthetic substrate *o*-nitrophenyl-β-glucopyranoside (Lenartovicz et al., 2003). In all cases, one unit of enzyme activity was defined as the amount of enzyme capable of releasing 1 μmol of product per min.

2.5. Saccharification of untreated and pretreated lignocellulosic materials

The enzymatic hydrolysis of untreated and pretreated eucalyptus sawdust and rice hulls was performed using the commercial enzyme cocktails Cellic® CTec2 and Cellic® HTec2 in the proportion 9:1 (Jung et al., 2013). The activities of endocellulase, β-glucosidase and endoxylanase in CTec2 (final activities) were 1084 U·mL^{-1}, 12,152 U·mL^{-1} and 2459 U·mL^{-1}, respectively. The activities of endocellulase, β-glucosidase and endoxylanase in HTec2 (final activities) were 841 U·mL^{-1}, 9 U·mL^{-1} and 8153 U·mL^{-1}, respectively. The saccharification was carried out in 50 mL Erlenmeyer flasks under agitation of 150 rpm for up to 48 h. The reaction med-