



# Effect of four pretreatments on enzymatic hydrolysis and ethanol fermentation of wheat straw. Influence of inhibitors and washing



Cristina Toquero, Silvia Bolado\*

Department of Chemical Engineering and Environmental Technology, University of Valladolid, Dr. Mergelina s/n, 47011 Valladolid, Spain

## HIGHLIGHTS

- Four pretreatments were compared for bioethanol production from wheat straw.
- Pretreatment efficiency is linked to structural changes in straw and lignin removal.
- Water washing removes inhibitory compounds generated during pretreatment.
- Basic pretreatment increases sugar release but also inhibitor production.
- Alkaline peroxide is an efficient wheat straw pretreatment for producing bioethanol.

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## ABSTRACT

Pretreatment is essential in the production of alcohol from lignocellulosic material. In order to increase enzymatic sugar release and bioethanol production, thermal, dilute acid, dilute basic and alkaline peroxide pretreatments were applied to wheat straw. Compositional changes in pretreated solid fractions and sugars and possible inhibitory compounds released in liquid fractions were analysed. SEM analysis showed structural changes after pretreatments. Enzymatic hydrolysis and fermentation by *Pichia stipitis* of unwashed and washed samples from each pretreatment were performed so as to compare sugar and ethanol yields. The effect of the main inhibitors found in hydrolysates (formic acid, acetic acid, 5-hydroxymethylfurfural and furfural) was first studied through ethanol fermentations of model media and then compared to real hydrolysates. Hydrolysates of washed alkaline peroxide pretreated biomass provided the highest sugar concentrations, 31.82 g/L glucose, and 13.75 g/L xylose, their fermentation yielding promising results, with ethanol concentrations reaching 17.37 g/L.

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## 1. Introduction

The threat of imminent global warming coupled with diminishing crude oil reserves worldwide and rising energy demands entail the urgent need to replace fossil-based fuels with green biofuels. Biomass-based fuels, such as bioethanol, provide a promising alternative, since their energy is already included in the global carbon cycle, implying a significant reduction in carbon dioxide release (Karagöz et al., 2012). Among biomass feedstock, lignocellulosic residues offer attractive renewable sources for bioethanol production as they do not compete with the food industry and represent the most abundant carbohydrate reserve worldwide (Saha et al., 2013). In many countries, including Spain, crop straw is a plentiful by-product from agricultural activities, wheat straw having been selected as the raw material in the present study.

Lignocellulosic materials are rigid structures consisting of a carbohydrate polymer matrix (mainly cellulose and hemicelluloses). They are cross-linked and strongly bound to lignin. This structural complexity, defined as biomass recalcitrance, severely restricts enzymatic and microbial accessibility (Pu et al., 2013). Efficient pretreatments are thus required to disrupt the heterogeneous matrix, increase surface area and porosity of the cellulosic material, and to unlock carbohydrates from their lignin association, thereby enhancing enzymatic digestibility. The physicochemical properties and behaviour of each lignocellulosic feedstock differ. Appropriate pretreatments based on the characteristics of each raw material thus need to be applied (Karagöz et al., 2012).

Talebnia et al. (2010) thoroughly reviewed the pretreatments more commonly applied to wheat straw, including physical pretreatments (size reduction), physico-chemical pretreatments (liquid hot water, steam explosion, ammonia fiber explosion), chemical pretreatments (acid, alkaline, alkaline/oxidative, wet oxidation, ozonolysis) and biological pretreatments. The present work

\* Corresponding author. Tel.: +34 983423958; fax: +34 983423013.

E-mail address: [silvia@iq.uva.es](mailto:silvia@iq.uva.es) (S. Bolado).

focuses on thermal, dilute acid, alkaline, and alkaline/oxidative pretreatments in order to compare the results and select the most suitable pretreatments for future detailed study and optimization.

Thermal pretreatments are effective for improving enzymatic hydrolysis and are considered eco-friendly processing technologies since no chemicals are added, thus avoiding corrosion problems and decreasing toxic compound formation (Saha et al., 2013). Thermal autoclaving requires low energy consumption and is easy to perform, results being reported with sweet sorghum bagasse (Cao et al., 2012b).

Dilute acid pretreatment has been widely applied, as it is low-priced, effectively hydrolyzes hemicelluloses into monomeric sugars and causes structural transformation of other biomass components, thereby increasing enzyme accessibility and improving cellulose conversion (Cao et al., 2012a). Various acids have been used under different conditions of time, temperature, pressure, and concentration, particularly H<sub>2</sub>SO<sub>4</sub> with, for example, wild sugarcane (Chandel et al., 2011) and sugar beet pulp (Zheng et al., 2013), and HCl with, for example, sorghum straw (Herrera et al., 2004) and wheat straw (Marcotullio et al., 2011). Other approaches have also been adopted using HNO<sub>3</sub> and H<sub>3</sub>PO<sub>4</sub> with, for example barley straw (Panagiotopoulos et al., 2009).

Applying alkaline solutions allows easy removal of lignin and xylan side chains, substantially enhancing enzymatic saccharification efficiency (Park et al., 2010). Sodium and calcium hydroxide are appropriate chemicals for this kind of pretreatment, and are commonly used under mild conditions. NaOH is widely used for lignocellulose pretreatment as it evidences high lignin removal and hemicellulose solubilisation by fracturing ester bonds, and thereby increasing the porosity of the biomass (Kang et al., 2012). Studies have been performed using different sources of lignocellulosic biomass, especially wheat straw (Akhtar et al., 2001) and rice straw (Oberoi et al., 2012).

Combining alkaline and oxidizing reagents proves highly effective in removing lignin and hemicellulose, providing a less structured material with increased porosity and less deteriorated polysaccharide chains, and thereby resulting in high sugar release after enzymatic hydrolysis of pretreated biomass (Monte et al., 2011). Alkaline peroxide pretreatment has successfully been developed for different lignocellulosic materials such as sweet sorghum bagasse (Cao et al., 2012b), rapeseed straw (Karagöz et al., 2012; Luo et al., 2011), and rice hulls (Díaz et al., 2013).

For optimal ethanol production, the goal is to maximize release of sugars and their corresponding fermentation into ethanol (Karagöz et al., 2012). *Pichia stipitis* can ferment both xylose and glucose with high ethanol yields, making it a highly recommendable option for fermenting lignocellulosic hydrolysates (Bellido et al., 2011), given the high percentage of pentoses in this type of biomass. In addition, several toxic compounds are usually formed during pretreatment as a result of lignin and sugar degradation, and they may affect the downstream hydrolysis and fermentation steps (Kont et al., 2013).

The presence and quantity of such compounds depend on the raw material as well as on the severity of pretreatment. Working at extreme pH favours the formation of these compounds, with thermal and acid pretreatments entailing a greater risk of the formation of furaldehydes (i.e. 5-hydroxymethylfurfural (HMF) and furfural) and aliphatic acids (i.e. formic and acetic acid), whereas alkaline and oxidative pretreatments are more likely to generate aromatic compounds (i.e. phenolics) as well as acetyl derivatives (Hendriks and Zeeman, 2009). Furthermore, the most commonly found carboxylic acids in hydrolysates are formic and acetic acid, the latter being a by-product from both enzymatic hydrolysis and fermentation processes.

The present study aims to compare the effects of four pretreatment methods vis-à-vis improving enzymatic hydrolysis of wheat

straw and ethanol production from hydrolysates, considering the influence of inhibitory compounds and the benefit of washing the pretreated biomass prior to saccharification and fermentation processes. Electronic microscopy was used for qualitative evaluation of pretreatment effect on wheat straw. Model solutions were used to study the inhibitory fermentation effect of four compounds typically produced during the pretreatments considered: formic acid, acetic acid, HMF, and furfural.

## 2. Methods

### 2.1. Materials

Wheat straw, provided by the Castilla & León Institute of Technological Agriculture in Valladolid (Spain), was size-reduced to pass a 3–5 mm sieve and was kept in an oven at 45 °C prior to pretreatments. National Renewable Energy Laboratory (NREL) analytical methods were followed to determine raw material composition in terms of structural carbohydrates and lignin (Sluiter et al., 2010). Raw material (hereinafter RM) had the following composition (in% w/w): cellulose, as glucose, 35.19 ± 0.29; hemicellulose, as xylose, 22.15 ± 0.21; acid lignin, 22.09 ± 0.31 (soluble 3.92 ± 0.39, insoluble 18.17 ± 3.21); ash, 7.49 ± 0.29; moisture, 6.02 ± 0.11.

Sodium hydroxide (pellets), hydrochloric acid (37%), and hydrogen peroxide (33%), from Panreac Quimica S.L.U., were used in the preparation of pretreatment solutions. D(+)-glucose anhydrous, D(+)-xylose, ethanol (96%), formic acid (98%), and acetic acid (99.5%), from Panreac Quimica S.L.U., and 5-hydroxymethylfurfural (HMF) (99%) and furfural (>99%) from Sigma Aldrich, were used as standards to prepare model solutions and to determine the composition of the liquid phase through high performance liquid chromatography (HPLC). Gallic acid monohydrate (>98%), Folin & Ciocalteu's phenol reagent and sodium carbonate (>99%) from Sigma Aldrich were used for total phenolics determination. MilliQ Ultra-pure water was used.

Enzymatic complexes NS50013 (cellulase) and NS50010 (β-glucosidase) were kindly donated by Novozymes (Denmark). Xylanase was not supplemented, as NS50013 complex has been reported to show xylanase activity of 55 U/mL (Zhao et al., 2011; Travaini et al., 2013).

### 2.2. Pretreatments of wheat straw

Four different thermal and thermochemical pretreatments were applied in this study: thermal autoclaving (A), dilute HCl solution autoclaving (B), dilute NaOH solution autoclaving (C), and alkaline peroxide (D), using previously published conditions. In autoclave pretreatments A, B, and C, milled and dry wheat straw was slurried for 5 min with distilled water (Cao et al., 2012b), 1.5% w/w HCl solution (Sun and Cheng, 2005), and 1% w/w NaOH solution (Akhtar et al., 2001), respectively, in a 500 mL screw cap bottle in a solid:liquid ratio of 1:10 w/w, and then autoclaved at 121 °C for 60 min. In alkaline peroxide pretreatment (D), milled and dry wheat straw was slurried for 5 min with 5% w/w H<sub>2</sub>O<sub>2</sub>, in a solid:liquid ratio of 1:20, the pH was then adjusted to 11.5 with 2 M NaOH and the mixture was placed in a rotatory shaker at 50 °C and 120 rpm for 60 min (Karagöz et al., 2012).

After cooling down to room temperature, the slurry obtained from each pretreatment was recovered and residual solid was separated by filtration and dried in an oven at 45 °C for 48 h. Liquid fraction was stored in a refrigeration chamber for composition analysis. Half of the dry solid fraction from each pretreatment was washed with distilled water in a solid:liquid ratio of 1:10, at 120 rpm, for 60 min at room temperature. It was then filtered and dried in an oven at 45 °C for 48 h. Samples from washing liquid

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