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Effect of inhibitors formed during wheat straw pretreatment on ethanol fermentation by *Pichia stipitis*

Carolina Bellido, Silvia Bolado, Mónica Coca, Susana Lucas, Gerardo González-Benito, María Teresa García-Cubero *

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

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ABSTRACT

The inhibitory effect of the main inhibitors (acetic acid, furfural and 5-hydroxymethylfurfural) formed during steam explosion of wheat straw was studied through ethanol fermentations of model substrates and hydrolysates from wheat straw by *Pichia stipitis*. Experimental results showed that an increase in acetic acid concentration led to a reduction in ethanol productivity and complete inhibition was observed at 3.5 g/L. Furfural produced a delay on sugar consumption rates with increasing concentration and HMF did not exert a significant effect. Fermentations of the whole slurry from steam exploded wheat straw were completely inhibited by a synergistic effect due to the presence of 1.5 g/L acetic acid, 0.15 g/L furfural and 0.05 g/L HMF together with solid fraction. When using only the solid fraction from steam explosion, hydrolysates presented 0.5 g/L of acetic acid, whose fermentations have submitted promising results, providing an ethanol yield of 0.45 g ethanol/g sugars and the final ethanol concentration reached was 12.2 g/L (10.9 g ethanol/100 g DM).

1. Introduction

Lignocellulosic materials are a promising alternative energy to fossil resources because they are the most abundant natural renewable organic material that exists on earth and there are concerns over CO_2 emissions from fossil fuels (Prasad et al., 2007).

Conversion process of lignocellulosic biomass to ethanol can be divided into five unit operations (Merino and Cherry, 2007). (1) Size reduction to increase surface area and uniformity, (2) pretreatment to disrupt lignin and hemicellulose, reduce cellulose crystallinity and increase the porosity of the biomass, (3) enzymatic hydrolysis to convert sugar polymers to monomeric sugars, (4) fermentation to produce ethanol from those monomeric sugars and (5) ethanol recovery using distillation or other separation technologies.

Lignocellulose is a matrix of cross-linked polysaccharide networks, mainly cellulose and hemicellulose, which are tightly bound to lignin (Zaldivar et al., 2001). The efficient use of the sugar content of lignocellulosic biomass is the key for the economic feasibility of ethanol production. This implies that in addition to the glucose obtained from the cellulosic portion, all sugars released from the hemicellulose fraction, such as xylose and non-structural sugars, which may represent a significant percentage of the fermentable sugars, should be fermented (Díaz et al., 2009). Hence, the total ethanol production can be increased using an efficient xylose-fermenting yeast that can convert both hexose and pentose sugars (Nigam, 2001). Among the xylose-fermenting yeasts, *Pichia stipitis* has shown the most promising results for industrial application, because it ferments xylose with a high ethanol yield and it is also able to ferment glucose. Besides, *P. stipitis* does not require vitamin addition for xylose fermentation and it is capable to ferment a wide range of sugars, including cellobiose (Agbogbo and Wenger, 2006).

On the other hand, some of the compounds formed during pretreatment of lignocellulosic biomass such as sugar decomposition products and lignin degradation products may have a potential inhibitory effect on the fermentation process. Inhibitory compounds in lignocellulosic hydrolysates comprise aliphatic acids (i.e. acetic, formic and levulinic acid), furaldehydes (i.e. furfural and 5-hydroxymethylfurfural (HMF)), aromatic compounds (i.e. phenolics) and extractives (Martín and Jönsson, 2003). The number and identity of these toxic compounds varies with the type of the raw material and pretreatment conditions. An extensive study about the effects of each pretreatment has been done recently (Hendriks and Zeeman, 2009). In this study, steam explosion, a leading pretreatment for wheat straw biomass, was used as a delignification and hemicellulose solubilization method (Tomás-Pejó et al., 2008).

The main objective of this work is to analyze the influence of inhibitors formed during steam explosion of lignocellulosic biomass on ethanol fermentation by *P. stipitis*. This study is focused on three inhibitors: acetic acid, 2-furaldehyde (furfural) and

* Corresponding author. Tel.: +34 983 42 32 37; fax: +34 983 42 36 16.
E-mail address: maite@iq.uva.es (M.T. García-Cubero).

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5-hydroxymethylfurfural (HMF) in a concentration range of (0.5–3.5 g/L), (0.5–2 g/L) and (0.1–0.5 g/L), respectively. Three different approaches were used: first, the inhibitory effect of acetic acid, furfural and HMF in a model substrate medium, was studied. Then, the fermentation of wheat straw hydrolysates was analyzed. Finally, the effect of steam explosion liquid addition and the influence of the presence of solid fraction during the fermentation process were also studied.

2. Methods

2.1. Microorganism and media

P. stipitis DSM 3651 was obtained from the German Collection of Microorganisms and Cell Cultures. The yeast was maintained on YEPX agar plates at 4 °C in a refrigeration chamber containing 10 g/L yeast extract, 20 g/L peptone, 20 g/L xylose and 20 g/L agar. The inoculum medium was prepared as follows: a solution of 10 g/L yeast extract and 20 g/L peptone was sterilized at 120 °C for 20 min in an autoclave. Carbon source, 20 g/L xylose was then added after being sterilized (0.20 μm Sterile Filters, Ministart Sartorius) and *P. stipitis* was supplemented from YEPX agar plates. Inoculum was grown aerobically on a rotatory shaker (WY-200, Comecta, S.A.) at 175 rpm and 30 °C for 24 h. Yeast initial concentration was 0.5 g/L in all experimental runs.

2.2. Fermentation with model substrate media

When inhibitor effect was studied individually, model fermentation medium was composed of 35 g/L glucose, 20 g/L xylose, 10 g/L yeast extract, 20 g/L peptone, 0.47 g/L $(NH_4)_2SO_4$, 12.8 g/L KH_2PO_4, 0.51 g/L Na_2HPO_4 and 0.47 g/L $MgSO_4{\cdot}7H_2O$. This medium was adjusted to pH 5 with a buffer composed of two solutions (mL/L fermentation medium): 250 mL succinic acid 0.2 M and 267 mL NaOH 0.2 M. The medium was autoclaved at 120 °C for 20 min, and sugars were added after being filtered (0.20 μm Sterile Filters, Ministart Sartorius).

Fermentation experiments were carried out in sterile 125 mL serum bottles with cap and needle to remove CO_2 in a rotatory shaker at 30 °C and 175 rpm for 168 h. Experiments were performed with no oxygen supply, but they were not strictly anaerobic because there was air before closing serum bottles. Each serum bottle was filled with 25 mL of fermentation inoculated with 10% (v/v) growth culture.

Inhibitor compounds (acetic acid, furfural and HMF) were added either separately to study their individual effect using typical inhibitors concentrations for wheat straw hydrolysates according to the literature (Delgenes et al., 1996; Díaz et al., 2009) or as a mixture to simulate similar sugars and inhibitors concentration of wheat straw hydrolysates. A fermentation experiment without inhibitors was also carried out as a control. Fermentation process was monitored for 7 days by taking samples at 0, 24, 48, 72, 96, 120, 144 and 168 h for analyses. Table 1 summarizes the initial sugars and inhibitors concentration for fermentations performed with model solutions. All the experiments were carried out in triplicate and the average data are shown.

2.3. Wheat straw hydrolysates

2.3.1. Raw material

Wheat straw used in experiments was provided by the Institute of Technological Agriculture of Castilla y León. Wheat straw was milled using a laboratory sieve mill into small particles of 20 mm prior to steam explosion pretreatment. Raw material was kept in an oven at 45 °C until use. Wheat straw had the following composition (% dry weight): cellulose, as glucose, 32.4; hemicellulose, as xylose, 19.1; acid lignin, 21.3; ash, 6.4; moisture, 6.9.

2.3.2. Wheat straw pretreatment and saccharification

Steam explosion was used as a delignification and hemicelluloses release method. Pretreatment assays were carried out in a 5 L stainless steel batch reactor equipped with a quick-opening valve and a controller for residence time and temperature. Wheat straw was exploded at 210 °C for 10 min. After pretreatment, the slurry was recovered and residual solid was separated by filtration. Liquid fraction was stored in a refrigeration chamber. Depending on the experiment, the whole slurry or just the solid fraction was used.

After steam explosion, enzymatic hydrolysis was performed using a mixture of cellulase (NS50013) and β-glucosidase (NS50010), 0.11 g/g cellulose and 0.05 g/g cellulose were added, respectively. Enzymes were kindly donated by Novozymes (Denmark). Hydrolysis was carried out at 50 °C for 72 h in a 250 mL stirred tank at 175 rpm with mechanical agitation (Heidolph RZR 2020). When solid fraction was used for experiments, pretreated material was suspended in 0.05 M citrate buffer (pH 5.0) with a solid content of 10% (w/v), while the whole slurry at the same solid loading was supplemented with a concentrated 0.5 M citrate buffer to avoid sugars dilution. Depending on the experiment, the whole hydrolysate or just the liquid fraction (separated by vacuum filtration) were used for fermentation process.

2.4. Fermentation of wheat straw hydrolysates

Experimental runs can be firstly divided into two groups of experiments after steam explosion. In order to analyze the influence of the addition of steam explosion liquid, two sets of experiments were carried out either with the whole slurry (WS) or just with the solid fraction (SF). In addition, to study the influence of solid fraction

Table 1
Initial sugars and inhibitors concentrations for synthetic fermentations.

Experiment	Initial sugars concentration (g/L)		Inhibitor concentration (g/L)		
	Glucose	Xylose	Acetic acid	Furfural	HMF
Control	36.5	21.0	0	0	0
HAc2.5	35.1	20.5	2.5	0	0
HAc1.5	35.0	20.5	1.5	0	0
HAc0.5	35.0	20.1	0.5	0	0
F2	35.8	20.2	0	2	0
F1	35.1	21.4	0	1	0
F0.5	34.8	21.3	0	0.5	0
HMF0.5	37.0	20.5	0	0	0.5
HMF0.1	36.7	20.2	0	0	0.1
Model FH-SF	22.3	5.8	0.5	0	0
Model FH-WS	21.4	11.1	1.5	0.15	0.05