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Research paper

Enzymatic hydrolysis and detoxification of lignocellulosic biomass are not always necessary for ABE fermentation: The case of *Panicum virgatum*

Ana I. Paniagua-García[a,b,*], María Hijosa-Valsero[a], Rebeca Díez-Antolínez[a,b], Marta E. Sánchez[b], Mónica Coca[c]

[a] Centre of Biofuels and Bioproducts, Instituto Tecnológico Agrario de Castilla y León (ITACyL), Villarejo de Órbigo, E-24358, León, Spain
[b] Chemical and Environmental Bioprocess Engineering Group, Natural Resources Institute (IRENA), Universidad de León, Avenida de Portugal 42, E-24071, León, Spain
[c] Department of Chemical Engineering and Environmental Technology, Universidad de Valladolid, C/Doctor Mergelina s/n, E-47011, Valladolid, Spain

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ABSTRACT

Hemicellulosic hydrolysate of switchgrass, pre-treated with dilute sulfuric acid, was assessed for butanol production via acetone-butanol-ethanol (ABE) fermentation. *Clostridium beijerinckii* CECT 508 was selected among eight wild strains as the most efficient to produce butanol from glucose/xylose mixtures. The effects of inhibitory compounds from the acid hydrolysate on ABE fermentation were studied using model fermentation media, observing that the most harmful inhibitors were acetic acid > phenolic compounds > sulfate > furfural, while 5-HMF and levulic acid seemed to have no effect. Several detoxification treatments, including evaporation, overliming and activated charcoal adsorption, were evaluated to remove inhibitors from switchgrass acid hydrolysate. Although activated charcoal was the most effective method, there were no significant differences in butanol production between non-detoxified and detoxified hydrolysates. The non-detoxified switchgrass acid hydrolysate (containing 26 g L^{-1} xylose, 4 g L^{-1} glucose and 4 g L^{-1} arabinose) was successfully fermented by *C. beijerinckii* CECT 508, obtaining 4.00 ± 0.71 g L^{-1} butanol (yield 0.184 ± 0.032 g g^{-1}). To the best of our knowledge, this is the first time that a hydrolysate obtained from switchgrass has been efficiently fermented to butanol without previous enzymatic hydrolysis or detoxification steps, using a non-genetically modified *Clostridium* strain.

1. Introduction

During the last years, there has been an increasing interest in the production of ethanol and butanol as alternative biofuels because of two fundamental reasons: the finite rate of fossil fuels and the environmental deterioration worldwide. Butanol has greater advantages compared to ethanol as a fuel, since it is more hydrophobic, has higher energy density and higher boiling point, and can be mixed with gasoline in any proportion without modification of the gasoline engines [1].

Butanol can be produced by anaerobic sugar fermentation from a variety of feedstocks (like lignocellulosic biomass) using *Clostridium* strains (e.g. *C. acetobutylicum* or *C. beijerinckii*). This process is known as acetone-butanol-ethanol (ABE) fermentation.

Lignocellulosic biomass (corn stover, switchgrass, wheat straw, forest wastes, etc.) is considered an abundant and cheap material and consists mainly of polysaccharides containing hexose and pentose sugars [2]. Switchgrass (*Panicum virgatum* L.) presents a series of advantages to be used as an energy crop, such as its high production yield and low needs for water and nutrients for its growth, which makes it suitable to be produced in lands considered of bad quality for other crops [3]. Clostridia strains are not able to use lignocellulose directly as a carbon source, instead of this, lignocellulose has to be hydrolyzed in order to obtain five and six-carbon sugars which can be metabolized by this bacterial group [4]. The hydrolysis of the biomass usually consists of a physical-chemical pretreatment (to alter the structure of lignocellulose and to increase cellulose digestibility) followed by a subsequent enzymatic hydrolysis [5]. The pretreatment choice is very important to achieve an efficient conversion of the lignocellulosic material into fermentable sugars [6].

Dilute acid pretreatment is one of the most used techniques due to its high efficiency and low cost. This kind of pretreatment dissolves hemicellulose releasing mainly pentoses, redistributes lignin and increases cellulose digestibility in the pretreated material [7]. For many lignocellulosic materials, such as switchgrass, hemicellulose constitutes one third of its total carbohydrates [7], therefore, the efficient use of sugars from the hemicellulose fraction is essential to maximize butanol

* Corresponding author. Centre of Biofuels and Bioproducts, Instituto Tecnológico Agrario de Castilla y León (ITACyL), Villarejo de Órbigo, E-24358, León, Spain.
E-mail address: pangaran@itacyl.es (A.I. Paniagua-García).

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production in an economically favorable process [8].

On the other hand, the main problem related to dilute acid pretreatment is the generation of fermentation inhibitory compounds [9]. These microbial toxic chemicals can be classified into four types: sugar degradation products, lignin degradation products, structural compounds of lignocellulose, and heavy metallic ions [10] and from these, carboxylic acids, aldehydes, furans and phenolics are the most common inhibitory compounds [11]. To overcome the inhibitory effect of these products present in acid hydrolysates, several detoxification methods (physical, chemical and biological) have been proposed. The composition of the hemicellulosic hydrolysate and the microbial species employed to ferment, determine the effectiveness of a detoxification method [12].

In this way, although in most of the processes the liquid fraction coming from the acid pretreatment is discarded, and only the solid biomass is further used, this liquid fraction can be used for ABE fermentation, especially when the lignocellulosic substrate contains a significant fraction of xylan [13]. The fermentation of hemicellulosic hydrolysates of corn fiber treated with dilute sulfuric acid has been reported for butanol production by *C. beijerinckii* IB4 (a mutant derived from *C. beijerinckii* NCIMB 8052) [14,15].

Regarding switchgrass, no studies on ABE fermentation from its hemicellulosic hydrolysate, based on dilute acid pretreatment, have been found in literature. Using this energy crop, only a few studies have been conducted to obtain butanol from enzymatic hydrolysates (with high glucose concentration), discarding the pretreatment hydrolysate, or mixing the enzymatic hydrolysate with the pretreatment liquid fraction. In this way, Gao et al. [16] and Liu et al. [17] produced butanol from switchgrass enzymatic hydrolysate pretreated by alkaline methods (NaOH) or hydrothermolysis, after discarding the pretreatment hydrolysate. In addition, Qureshi et al. [18] have reported butanol production from dilute sulfuric acid pretreated switchgrass in a process which included enzymatic hydrolysis of the acid pretreated solid biomass without separation of the liquid fraction released in the pretreatment. Butanol production after ABE fermentation was low (0.97 g L^{-1} and butanol yield 0.054 g g^{-1}) and remained low after overliming detoxification, but it was remarkably improved after dilution of the hydrolysate with water and glucose addition (9.55 g L^{-1} butanol, with a butanol yield of 0.255 g g^{-1}).

The present study exposes a new approach based on the utilization of the liquid fraction from switchgrass acid pretreatment to assess butanol production using wild strains of *C. beijerinckii*. The objectives of this study were: (i) to select a suitable bacterial strain to perform ABE fermentation on this hydrolysate, (ii) to study the effect of inhibitors from switchgrass acid hydrolysate on ABE fermentation, and (iii) to evaluate the necessity and effectiveness of several detoxification techniques to remove inhibitory compounds and improve butanol production. To the best of our knowledge, this is the first time that switchgrass acid hydrolysate is efficiently fermented to produce butanol without the necessity of a previous detoxification treatment and using a non-genetically modified strain of *C. beijerinckii*.

2. Material and methods

2.1. Chemicals and reagents

Sugars (glucose, xylose and arabinose), chemical reagents for use in analytical methods (acetic acid, butyric acid, *p*-coumaric acid, ferulic acid, gallic acid, levulinic acid, acetone, butanol, ethanol, 5-hydroxymethylfurfural (5-HMF), furfural and Folin-Denis' reagent) were purchased from Sigma-Aldrich Chemicals (St. Louis, MO, USA). Other chemicals were of reagent grade and were obtained from two suppliers: $FeSO_4{\cdot}7H_2O$, $MgSO_4{\cdot}7H_2O$, NH_4Cl, $CaCO_3$, $CaCl_2$, $Ca(OH)_2$, yeast extract and cysteine from Sigma-Adrich Chemicals, and HCl, H_2SO_4, NaOH, CaO, K_2HPO_4, KH_2PO_4, Na_2CO_3 from Panreac (Castellar del Vallès, Barcelona, Spain). Compressed gasses (N_2, He, H_2 and air) used in gas chromatography and to generate anaerobic conditions to produce ABE fermentation, were obtained from Contse, S.A. (Madrid, Spain).

2.2. Lignocellulosic biomass

Switchgrass (*P. virgatum*), cv. *Alamo*, was used as feedstock for ABE production. Switchgrass was grown in a test plot of ITACyL (Agrarian Technologic Institute of Castile and Leon) located in Valladolid (Spain). The material was harvested at the beginning of November 2013. The feedstock was dried in an oven at 45 °C for 48 h, ground in a SM100 Comfort rotary mill (Retsch GmbH, Haan, Germany) and passed through a 1.0 mm screen [19]. Milled material was stored at room temperature in airtight containers until being used. The compositional analysis of the untreated switchgrass (dry basis) was: 33.0 ± 0.4% glucan, 21.4 ± 0.4% xylan, 2.4 ± 0.4% arabinan, 20.3 ± 1.2% acid insoluble lignin, 4.6 ± 0.2% acid soluble lignin and 7.0 ± 0.4% ash [6]. The glucan, xylan, arabinan, lignin and ash contents of switchgrass were in agreement with values reported by other authors [16,17].

2.3. Acid hydrolysis

The dilute sulfuric acid pretreatment of switchgrass was performed in 1-L screw-capped bottles at optimum conditions previously determined [6], in order to release the maximum amount of sugars and the minimum amount of inhibitors in the hydrolysate. Briefly, dilute sulfuric acid at 1.72% (w/w) was used to treat milled switchgrass at a solid loading of 10.0% (w/w) in an autoclave at 121 °C (103 kPa) during 112 min. After the pretreatment, the suspension was cooled down and the acid hydrolysate was collected by vacuum filtration using a Büchner funnel with cellulose filters (20–25 μm, Model 1238, Filter Lab, Barcelona, Spain). The chemical composition of the liquid hydrolysate is shown in Table 1.

2.4. Bacterial strains and culture conditions

Eight strains of *Clostridium beijerinckii* were screened, namely, CECT 508 (NCIMB 8052) (CECT, Paterna, Spain), DSM 51, DSM 552, DSM 791, DSM 1820, DSM 6422, DSM 6423 and DSM 13821 (DSMZ, Braunschweig, Germany). Strain culture was performed according to Díez-Antolínez et al. [20]. In brief, lyophilised cells were resuspended in Reinforced Clostridial Medium broth (Oxoid, Basingstoke, UK) supplemented with 10 g L^{-1} glucose (Sigma-Aldrich, Steinheim, Germany) and incubated at 35 °C during 10 days. Subsequently, they were subjected to sporulation according to CECT protocol. Laboratory stocks of strain spores were stored in sterile H_2O at 4 °C. Then, 125 μL spores were added to 50 mL of the above-mentioned culture medium, placed in glass bottles capped with a rubber septum, and exposed to a thermal shock (2 min at 80 °C in a water bath and 5 min in ice). Afterwards,

Table 1
Chemical composition of the switchgrass acid hydrolysate.

Compound	Concentration (g L^{-1})	Metal/Anion	Concentration (mg L^{-1})
Glucose	3.94 ± 0.11	Potassium	360
Xylose	25.99 ± 0.55	Sodium	47
Arabinose	3.88 ± 0.25	Calcium	195
Acetic acid	3.34 ± 0.18	Magnesium	163
Levulinic acid	0.30 ± 0.03	Copper	n.d.
5-HMF	0.20 ± 0.02	Iron	15
Furfural	0.59 ± 0.07	Manganese	3
Total phenolic compounds	1.03 ± 0.04	Zinc	1
Sulfate	16.87 ± 0.24	Chloride	252
		Nitrate	21
		Phosphate	328

n.d. Not detected.