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Development of dilute sulfuric acid pretreatment method for the enhancement of xylose fermentability

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ABSTRACT

Dilute acid pretreatment of sorghum stalks was performed for the characteristic xylulosic ethanol production using *Pichia stipitis*. In this regard, various pretreatment parameters were studied for the maximum xylose production with minimized xylose decomposition. As a result, 89% of xylan conversion was attained along with 22 mg of xylose decomposition at 121 °C with 0.2 M sulfuric acid for 120 min. In addition to this, prehydrolyzate liquors were detoxified with calcium hydroxide [$Ca(OH)_2$] at different pH levels (i.e., 8.5–11.5) to investigate the fermentative inhibitors removal and their impact on xylulosic ethanol production. Consequently, the highest theoretical ethanol yield (91%) was achieved during the fermentation of prehydrolyzate detoxified at pH 11.5. Moreover, 0.46 g_p/g_s ethanol yield and 0.48 g/L/h ethanol productivity was achieved within 24 h incubation time of *Pichia stipits* NCIM 3498.

1. Introduction

Lignocellulosic biomass is a renewable energy resource for the production of bioethanol which is an alternative solution to conquer the fossil fuels depletion. Lignocellulosic materials are relatively cheap and highly abundant in nature. These are mainly agricultural by-products such as sugarcane bagasse, corn stover, and wheat straw or forestry residues, which account for major sources of bioethanol production. Apart from these, sorghum stalks have a rich content of fermentable sugars which have been of particular interest for biofuels production (Schaffert, 1995). Sorghum is a C4 plant and is highly tolerant to drought than other crops (Prakasham et al., 2014). Consequently, it can be cultivated in tropical, subtropical and arid regions (Sree et al., 1999). Therefore, sorghum is considered as one of most promising large scale energy producing crop.

The complex structure of lignocellulosic materials consists of cellulose, hemicellulose and lignin. Cellulose is a homo-polymer which is made up of glucose units, whereas hetero-polymeric structure of hemicellulose is made up of xylose, arabinose and organic acids. Pretreatment should be the first step to unlock the lignocellulosic complex structure to release the monomeric sugars from their respective polymeric carbohydrates. In this regards, dilute-acid pretreatment is the most widely used and is proven to be a fast as well as cost effective method. This process significantly hydrolyzes the hemicellulose to yield pentose sugars along with a few other sugars from the lignocellulosic material (Gray et al., 2006). Xylose is a principle sugar formed during dilute acid pretreatment. The main disadvantage of dilute acid pretreatment was sugar degradation which leads to fermentative inhibitors formation such as furans (Furfural, 5-hydroxymethyl furfural) and then converted into organic acids (formic acid and levulinic acid) (Taherzadeh et al., 1999). Along with this, acid soluble lignin derived phenolic compounds were also formed (Palmqvist and Hahn-Hagerdal, 2000b). These compounds show inhibition effect on microbial growth during the fermentation process (Larsson et al., 1999). Therefore, conditioning of prehydrolyzates (pretreatment derived hydrolyzates) is an essential step which is employed prior to the successful fermentation for xylulosic ethanol production.

Several methods have been well established for the conditioning of prehydrolyzates. These include overliming, ion-exchange, steam stripping, treatment with activated carbon, solvent extraction, and microbial acclimation to improvise the fermentation efficiency of prehydrolyzates (Frazer and McCaskey, 1989; Gong et al., 1993; McMillan, 1994). Among them, overliming with calcium hydroxide is the most economical and widely used method for detoxification which assists in removal of furans such as furfural and 5- hydroxymethyl furfural (HMF) from the prehydrolyzates (Martinez et al., 2000; Ranatunga et al.,

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2000). However, certain limitations have been reported in overliming process i.e., sugar loss is generally caused when the pH is significantly elevated (Martinez et al., 2000; Nilvebrant et al., 2003) and also it is not an effective way to reduce toxicity caused by organic acids such as formic acid and acetic acid (Larsson et al., 1999; Palmqvist and Hahn-Hagerdal, 2000a; Ranatunga et al., 2000).

However, xylulosic ethanol production at high yield from prehydrolyzates would be helpful for the commercialization of lignocellulosic biofuels production process (Wyman, 2003). A number of naturally occurring yeast species such as *Candida shehatae*, *Pichia stipitis*, and *Pachysolen tannophilus* are able to efficiently ferment both glucose and xylose into ethanol (Parekh and Wayman, 1986; Schneider et al., 1981). Among them, *Pichia stipitis* exhibits good potential for industrial application, because it ferments xylose with a high ethanol yield (Agbogbo et al., 2006; du Preez and Prior, 1985). Therefore, the present study focused on sorghum biomass pretreatment method development with dilute sulfuric acid at stipulated parameters for maximum xylan conversion with minimized xylose decomposition. Additionally, validation of developed pretreatment process was done by fermentation of conditioned hydrolyzate using *Pichia stipitis*.

2. Materials and methods

2.1. Feedstock

Sorghum stalks were collected from experimental farm of International Crop Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, Hyderabad, Telangana, India. Biomass was washed with tap water to remove sand particles. The dried sorghum biomass was cut into pieces and then ground to obtain particle size range between 100 to 150 μm. This biomass was soxhlet extracted with water (12 h) and ethanol (8 h) for the removal of extractives (Sluiter et al., 2005). The structural carbohydrates composition of extractive free biomass was performed according to the National Renewable Energy Laboratory (NREL) procedure (Sluiter et al., 2011) and are listed in Table 1.

2.1.1. Dilute sulfuric acid pretreatment

Sorghum stalks pretreatment was performed with different sulfuric acid concentrations (i.e., 0.01 M, 0.1 M, 0.2 M and 0.4 M) for 2 h reaction time at 121 °C, with 5% (w/v) solid loading. At every 30 min interval, 0.1 mL of aliquot was collected from reaction mixture and used for quantification of carbohydrates and fermentative inhibitors by high-performance liquid chromatography (HPLC). At optimum xylan hydrolysis condition, solid and liquid fractions were separated through 0.2 μm nylon membrane filter. The solid fraction was washed with distilled water to attain neutral pH and then dried at 45 ± 3 °C, 48 h and subjected to compositional analysis according to the NREL procedure (Sluiter et al., 2011). Liquid fraction (denoted as unconditioned prehydrolyzate) was stored at −20 °C until the detoxification process.

Table 1
Chemical composition of sorghum biomass.

Composition	Untreated (%)[a]	Treated (%)[a]
Water extractives	12.1	–
Ethanol extractives	2.92	–
Cellulose	36.3	58.2
Hemicellulose	22.2	2.44
Xylose	*19.7*	*2.17*
Arabinose	*2.5*	*0.26*
Lignin	18.2	32.6

-, Not applicable.
[a] Composition percentages as on oven dry weight basis.

2.1.2. Detoxification of prehydrolyzates

During the detoxification process, the unconditioned prehydrolyzate was divided into four fractions and each fraction was treated with calcium hydroxide [$Ca(OH)_2$] to attain different pH levels i.e., 8.5, 9.5, 10.5 and 11.5. First, the unconditioned prehydrolyzates were heated to 50 °C and held at this temperature for 15 min. This was followed by slow addition of $Ca(OH)_2$ to reach the different targeted pH ranges and then continue the agitation for 30 min. These overlimed hydrolyzates were centrifuged to separate the calcium sulfate ($CaSO_4$) sludge and the supernatants pH was adjusted to 6 (which is a cultivation pH of *P. stipitis*) with 10 N sulfuric acid. Hereafter, these liquors are considered as conditioned hydrolyzates. These conditioned hydrolyzates were concentrated under reduced pressure by using rotary evaporator (Buchi, Rotavapor-R-210, and Switzerland) and then filter sterilized for xylulosic ethanol production studies.

2.2. Microorganism

Pichia stipitis NCIM 3948 (Same as CBS 6054) strain was procured from National Collection of Industrial Microorganisms (NCIM) Pune, India. This strain was supplied on MGYP agar slants, which contained (g/L): 3, Malt extract; 10, Glucose; 3, Yeast extract; 5, peptone; and 20, agar; and, pH was maintained at 6.4–6.8.

2.2.1. Seed culture preparation

Modified YPDX media was prepared based on the ratio of glucose and xylose (i.e., 1:4) present in the conditioned hydrolyzate. The composition of modified YPDX media includes (g/L): yeast extract-10, peptone-20, glucose-5, xylose-15 and agar-20 were autoclave sterilized. *P. stipitis* was cultivated on modified YPDX agar plates and incubated at 30 °C for 48 h. A colony from the plate was inoculated into a 250 mL Erlenmeyer flask containing 100 mL of filter sterilized YPDX liquid growth medium and incubated in a shaking incubator with agitation speed of 120 rpm at 30 °C for 18 h. The pH of the YPDX medium was maintained at 6. The cells were harvested by centrifugation at 8000 rpm for 10 min and re-suspended in sterile distilled water to adjust the final concentration of 40 g/L which is served as inocula for bioethanol production. Cell growth was observed by measuring the absorbance at 600 nm (OD_{600}) using UV–Visible spectrophotometer (Agilent, Cary 100, USA).

2.2.2. Fermentation

The fermentation was performed in sterile 25 mL Erlenmeyer flasks containing 10 mL of fermentation medium which includes 0.2 mL of 50X concentrated nutrient solution (1.7 g of yeast nitrogen base, 1 g of urea and 6.56 g of peptone in 20 mL of water), 0.5 mL of inocula gives an initial cell concentration of 2 g/L and, added an appropriate quantity of hydrolyzate to reach desired volume. Initial pH of the media was maintained at 6 and incubated at 30 °C with 120 rpm. All fermentation samples were taken periodically for HPLC analysis.

2.3. Analytical methods

Each sample was filtered through a 0.2 μm filter and appropriate dilution was made with Milli Q water. Sugars, fermentative inhibitors and ethanol quantitative analysis was performed using Varian 210 HPLC system with refractive index (RI) detector (355) (Varian, The Netherlands). The separation was achieved by Meta-carb −87 H carbohydrate column (300 × 6.5 particle size 8 μm) and was maintained at 60 °C with 9 mM sulfuric acid used as an eluent at a flow rate of 0.5 mL/min. RI detector was maintained at 50 °C for all the compounds. The total phenolic content present in the samples were determined by the Folin–Ciocalteu method (Singleton et al., 1999) with gallic acid as a standard.