Bioresource Technology 100 (2009) 1214–1220

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Separate hydrolysis and fermentation (SHF) of *Prosopis juliflora*, a woody substrate, for the production of cellulosic ethanol by *Saccharomyces cerevisiae* and *Pichia stipitis*-NCIM 3498

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ARTICLE INFO

Article history:
Received 19 April 2008
Received in revised form 20 August 2008
Accepted 20 August 2008
Available online 2 October 2008

Keywords:
Lignocellulosic biomass
Prosopis juliflora
Acid hydrolysis
Enzymatic hydrolysis
Fermentation

ABSTRACT

Prosopis juliflora (Mesquite) is a raw material for long-term sustainable production of cellulosics ethanol. In this study, we used acid pretreatment, delignification and enzymatic hydrolysis to evaluate the pretreatment to produce more sugar, to be fermented to ethanol. Dilute H_2SO_4 (3.0%, v/v) treatment resulted in hydrolysis of hemicelluloses from lignocellulosic complex to pentose sugars along with other byproducts such as furfural, hydroxymethyl furfural (HMF), phenolics and acetic acid. The acid pretreated substrate was delignified to the extent of 93.2% by the combined action of sodium sulphite (5.0%, w/v) and sodium chlorite (3.0%, w/v). The remaining cellulosic residue was enzymatically hydrolyzed in 0.05 M citrate phosphate buffer (pH 5.0) using 3.0 U of filter paper cellulase (FPase) and 9.0 U of β-glucosidase per mL of citrate phosphate buffer. The maximum enzymatic saccharification of cellulosic material (82.8%) was achieved after 28 h incubation at 50 °C. The fermentation of both acid and enzymatic hydrolysates, containing 18.24 g/L and 37.47 g/L sugars, with *Pichia stipitis* and *Saccharomyces cerevisiae* produced 7.13 g/L and 18.52 g/L of ethanol with corresponding yield of 0.39 g/g and 0.49 g/g, respectively.

1. Introduction

Worldwide high demand for energy, uncertainty of petroleum resources and concern about global climatic changes has led to the resurgence in the development of alternative liquid fuels. Ethanol has always been considered a better choice as it reduces the dependence on reserves of crude oil and promises cleaner combustion leading to a healthier environment. Developing ethanol as fuel beyond its current role of fuel oxygenate, would require lignocellulosics as a feedstock because of its renewable nature, abundance and low cost (Saha et al., 2005).

Lignocelluloses are mainly comprised of cellulose, a polymer of six-carbon sugar, glucose; hemicellulose, a branched polymer comprised of xylose and other five-carbon sugars and lignin consisting of phenyl propane units. The presence of lignin limits the fullest usage of cellulose and hemicellulose. To convert these energy rich molecules into simpler forms, it is necessary to remove the lignin from lignocellulosic materials. A number of pretreatments such as concentrated acid hydrolysis (Liao et al., 2006), dilute acid hydrolysis (Cara et al., 2008), alkali treatment (Carrillo et al., 2005), sodium sulphite treatment (Kuhad et al., 1999; Kapoor et al., 2008), sodium chlorite treatment (Sun et al., 2004), steam explosion (Ohgren et al., 2005), ammonia fiber explosion (Teymouri et al., 2005) lime treatment (Kim and Holtzapple, 2005), and organic solvent treatment (Xu et al., 2006) have been used frequently to remove lignin and improve the saccharification of the cell wall carbohydrates.

Of these methods, dilute acid treatment and enzymatic hydrolysis have been the most popular ones. Dilute acid hydrolysis is a fast and convenient method to perform but it leads to the accumulation of fermentation inhibitory compounds such as furfurals, hydroxy methyl furfurals (HMF) and phenolics. These compounds, depending on their concentration in the fermentation media, can inhibit microbial cell and affect the specific growth rate and cell-mass yield. Several treatments e.g., ion exchange (Canilha et al., 2004; Chandel et al., 2007), overliming (Martinez et al., 2001; Chandel et al., 2007), activated charcoal adsorption (Mussatto et al., 2004; Canilha et al., 2004; Chandel et al., 2007), and laccase oxidation treatment (Chandel et al., 2007) have been reported for the detoxification of hydrolysate to improve the fermentability of acid hydrolysates into ethanol. However, the combination of pH adjustment by overliming followed by activated charcoal adsorption has been shown to improve the detoxification of hemicellulosic hydrolysate (Converti et al., 1999).

The acid hydrolysis pretreatment removes the hemicellulosic portion and some fraction of lignin but rest of the lignin remains intact to the cellulosic substrate. Kaya et al. (2000) had reported that during enzymatic hydrolysis of lignocellulosic biomass,

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doi:10.1016/j.biortech.2008.08.033

cellulase components, β-glucosidase and endoglucanase have more binding affinity towards lignin than to the carbohydrates, resulting in lower efficiency of saccharification. Hence, to achieve maximum hydrolysis of cellulosics, which is a prerequisite for ethanol fermentation, an appropriate delignification treatment of biomass is required. In the present work, the combination of sodium sulphite and sodium chlorite for the delignification of cellulosic biomass has been attempted.

The cellulosic and hemicellulosic sugars obtained through acid and enzymatic hydrolysis can efficiently be used for ethanol fermentation either by separate fermentation of individual hydrolysate or fermentation of mixed hydrolysate using co-culture. However, in co-culture cultivation, optimum growth conditions of the yeasts would be different and might result in lower efficiency and lower product yield. Hence, for better efficiency of ethanol production, the approach of separate hydrolysis and fermentation (SHF) was preferred (Olsson and Hahn-Hagerdal, 1993).

In the present study, *Prosopis juliflora* (Mesquite), a perennial deciduous thorny shrub, the common vegetation of semi-arid region of Indian subcontinent, was used as a raw material for the production of cellulosic ethanol. The mesquite has recently been suggested to be used as raw material for long-term sustainable production of cellulosic ethanol (Hopkins, 2007). Its nature to tolerate drought, grazing, heavy soil, sand as well as saline dry flats and no competence with animal feed demand made it a potential low value substrate for ethanol production. Here, an attempt was made to saccharify *P. juliflora* into reducing sugars and eventually to ethanol fermentation.

2. Methods

2.1. Raw material and chemicals

Prosopis juliflora wood, collected from University of Delhi South Campus, New Delhi, India, was comminuted by a combination of chipping and milling to attain a particle size of 1–2 mm using a laboratory knife mill (Metrex Scientific Instrumentation, Delhi, India). The processed substrate was washed thoroughly and dried overnight at 60 °C.

Commercial cellulase from *Trichoderma reesei* (ATCC 26921) (6.5 FPU/mg), β-glucosidase (Novozyme 188) (250 U/g) from *Aspergillus niger* and 3,5-dinitrosalicylic acid (DNS) were purchased from Sigma, St. Louis, Missouri, USA. Ethanol was purchased from Merck (Darmstadt, Germany). Rest of the chemicals and media components of highest purity grade were purchased locally.

2.2. Micro-organisms and culture conditions

Pichia stipitis NCIM 3498 was procured from National Chemical Laboratory (NCL), Pune, India and was maintained on agar slants containing (g/L): xylose, 20.0; yeast extract, 4.0; peptone, 5.0; KH_2PO_4, 1.5; $MgSO_4 \cdot 7H_2O$, 0.5; agar, 20.0 at pH 5.0 ± 0.2 and temperature 30 °C. *Saccharomyces cerevisiae*, was procured from the culture collection of University of Delhi South Campus, New Delhi, India, and maintained on agar slants containing (g/L): glucose, 30.0; yeast extract, 3.0; peptone, 5.0; agar, 20.0 at pH 6.0 ± 0.2 and temperature 30 °C.

Inoculum of *P. stipitis* was prepared as described by Nigam (2001) using (g/L): xylose, 50.0; yeast extract, 3.0; malt extract, 3.0; peptone, 5.0 at pH 5.0 ± 0.2 and temperature 30 °C. *Saccharomyces. cerevisiae* inoculum was grown for 24 h at 30 °C in a culture medium containing (g/L): glucose, 30.0; yeast extract, 3.0; peptone, 5.0; $(NH_4)_2HPO_4$, 0.25 at pH 6.0 ± 0.2 (Chen et al., 2007). Cells were cultured to an optical density of 0.6–0.8 at 620 nm.

2.3. Proximate chemical composition analysis of the substrate

The chemical composition of *P. juliflora* wood was analysed for holocellulose, Klason lignin, pentosans, ash and moisture content. The plant material was extracted with alcohol–benzene (1:2 v/v) to remove wax, resin etc. The extractive-free wood dust was processed for chemical analysis following the TAPPI (1992) protocols, (α-Cellulose–TAPPI Method T203 om–83; Klason lignin–TAPPI Method T222 om–83; Pentosans–TAPPI Method T223 hm–84; Moisture–TAPPI Method T208 om–84 and Ash–TAPPI method 211om–93).

2.4. Dilute acid pretreatment

The dilute sulphuric acid pretreatment of wood dust was optimized at varied temperatures (100–140 °C), treatment time (15–60 min) and acid concentrations (1.0–5.0%, v/v) at 10.0% (w/v) consistency, in a 20.0 L plastic vessel (Carboy, Tarson Pvt. Ltd., Kolkata, India), using an autoclave (Russian make). The acid hydrolysate after treatment was recovered by filtering the contents through double-layered muslin cloth. The remaining wood dust was washed with tap water till neutral pH. The hydrolysate was analysed for sugars, phenolics, acetic acid and furans and the leftover plant biomass was dried overnight till constant weight and used for further experiments.

2.5. Detoxification of acid hydrolysate

The acid hydrolysate was overlimed at room temperature by adding dried lime [$Ca(OH)_2$] till the pH reached 10.0, with constant stirring for 30 min by an overhead stirrer (Remi Motors Ltd, Mumbai, India). After overliming, the hydrolysate was neutralized with concentrated H_2SO_4 and centrifuged at 10,000*g* for 15 min to remove the precipitate formed during neutralization. The overlimed hydrolysate was further detoxified by treating with activated charcoal (1.5%, w/v) with constant stirring at room temperature for 30 min and the sugar syrup was recovered through vacuum filtration.

2.6. Chemical delignification of acid pretreated woody biomass

Acid pretreated residue of *P. juliflora* was delignified by treating with sodium sulphite (5.0–20.0%, w/v) alone and combination of sodium sulphite (5.0%, w/v) and sodium chlorite (3.0%, w/v) and autoclaved at different temperatures (100–140 °C) for different time intervals (15–60 min). The delignified material was then filtered through double-layered muslin cloth and the leftover cellulosic residue was washed repeatedly with water till neutral pH. The cellulosic residue was dried overnight at 60 °C till constant weight and hydrolyzed enzymatically.

2.7. Enzymatic hydrolysis of delignified cellulosic substrate

Enzymatic hydrolysis of cellulose (delignified acid treated plant material) was carried out at a 5.0% (w/v) consistency in 0.05 M citrate phosphate buffer (pH 5.0) containing 0.005% sodium azide. Before enzyme loading, slurry was acclimatized by incubating at 50 °C on a rotatory shaker (Innova-40, New Brunswick Scientific, Germany) at 150 rpm for 2 h. Thereafter, a mixture of 3.0 U of filter paper cellulase (FPase) and 9.0 U of β-glucosidase per mL of citrate phosphate buffer was added to preincubated cellulose slurry and reaction continued for 36 h. Samples were withdrawn at regular interval of 4 h, centrifuged at 10,000*g* for 15 min and the supernatant was analysed for total reducing sugars released. The extent of hydrolysis was calculated as follows: