Bioresource Technology 101 (2010) 8348–8354

Contents lists available at ScienceDirect

Bioresource Technology

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

Bioethanol production from *Lantana camara* (red sage): Pretreatment, saccharification and fermentation

Ramesh Chander Kuhad [a,*], Rishi Gupta [a], Yogender Pal Khasa [a], Ajay Singh [b]

[a] Department of Microbiology, University of Delhi South Campus, Benito Juarez Road, New Delhi 110021, India
[b] Lystek International Incorporation, 107-279 Weber Street North, Waterloo, Ontario, Canada N2J3H8

ARTICLE INFO

Article history:
Received 18 March 2010
Received in revised form 26 May 2010
Accepted 7 June 2010
Available online 2 July 2010

Keywords:
Lantana camara
Cellulosic ethanol
Dilute acid hydrolysis
Enzymatic hydrolysis
Fermentation

ABSTRACT

Lantana camara contains 61.1% (w/w) holocellulose and can serve as a low-cost feedstock for bioethanol production. Acid hydrolysis (3.0%, v/v H_2SO_4, 120 °C for 45 min) of *L. camara* produced 187.14 mg/g total sugars along with fermentation inhibitors such as phenolics (8.2 mg/g), furfurals (5.1 mg/g) and hydroxy methyl furfurals (6.7 mg/g). Sequential application of overliming (pH 10.0) and activated charcoal (1.5%, w/v) adsorption was used to remove these toxic compounds from the acid hydrolysate. The acid-pretreated biomass of *L. camara* was further delignified through combined pretreatment of sodium sulphite (5.0% w/v) and sodium chlorite (3.0% w/v), which resulted in about 87.2% lignin removal. The enzymatic hydrolysis of delignified cellulosic substrate showed 80.0% saccharification after 28 h incubation at 50 °C and pH 5.0. Fermentation of acid and enzymatic hydrolysates with *Pichia stipitis* and *Saccharomyces cerevisiae* gave rise to 5.16 and 17.7 g/L of ethanol with corresponding yields of 0.32 and 0.48 g/g after 24 and 16 h, respectively.

1. Introduction

Lantana camara, commonly known as red sage, is one of the world's top 100 worst invasive species. It has invaded millions of hectares of grazing land globally (Day et al., 2003). In Australia alone, since its introduction as an ornamental plant in the 1840's it has spread to infest four million hectares (www.weeds.org.au). While in India, the weed has invaded most of the tropical and subtropical parts and is found in areas from the seacoast to 5000 ft in altitude (Sankaran, 2007). The approximate total biomass produced by *L. camara* per year ranges from 15 to 17 tonnes/ha (Bhatt et al., 1994), which projects the availability of *Lantana* biomass in huge quantity. Therefore, the abundance of *L. camara* biomass (lignocellulosic material) is likely to offer a potential feed stock for ethanol production.

Currently, the biomass to ethanol conversion technology relies mainly on chemical and enzymatic treatments. Chemical hydrolysis of biomass with dilute sulphuric acid has long been recognized as a critical step for removing the hemicellulosic fraction from the lignocellulosic substrate to economize the biological conversion of cellulosic biomass to ethanol. However, the pentose sugar-rich acid hydrolysate also contains toxic byproducts such as furfural, hydroxy methyl furfural (HMF) and phenolics, which significantly affect yeast cell metabolism during fermentation (Palmqvist and Hahn-Hagerdal, 2000; Chandel et al., 2007). Although various detoxification methods have been investigated for the removal of fermentation inhibitory compounds (Palmqvist and Hahn-Hagerdal, 2000; Chandel et al., 2007), of which overliming and activated charcoal adsorption methods are widely used (Miyafuji et al., 2003; Gupta et al., 2009).

An appropriate delignification strategy is essential for the efficient enzyme hydrolysis of cellulosic biomass as lignin hinders the saccharification process. The cellulase components such as β-glucosidase and endoglucanase showed higher binding affinity towards lignin compared to carbohydrates, which in turn lowered the saccharification efficiency (Kaya et al., 2000). Various delignification approaches have been exploited in the past such as alkali pretreatment (Carillo et al., 2005), hydrogen peroxide pretreatment (Saha and Cotta, 2007), sulphite pretreatment (Kuhad et al., 1999), ammonia fiber expansion pretreatment (Teymouri et al., 2005) and sodium chlorite pretreatment (Gupta et al., 2009).

The efficient conversion of biomass into ethanol requires optimum utilization of both pentose and hexose sugars. The widely studied yeast for hexose fermentation, *Saccharomyces cerevisiae*, is unable to utilize pentose sugars while, among the pentose fermenting yeasts only *Pichia stipitis*, *Pachysolen tannophilus* and *Candida shehatae* are proven to be highly efficient in the conversion of xylose to ethanol (Abbi et al., 1996a,b). The fermentation of both types of sugars can be carried out either by co-cultivation or

* Corresponding author. Address: Lignocellulose Biotechnology Laboratory, Department of Microbiology, University of Delhi South Campus, Benito Juarez Road, New Delhi 110021, India. Tel.: +91 124 24112062; fax: +91 11 24115270.
E-mail address: kuhad85@gmail.com (R.C. Kuhad).

0960-8524/$ - see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biortech.2010.06.043

through separate fermentation. Co-cultivation has several drawbacks such as the diauxic phenomenon (inefficient utilization of xylose in presence of glucose) (Grootjen et al., 1991), and competition between organisms for nutrients requirements. Recently some studies on separate fermentation of individual hydrolysates using sugar specific yeast strains have been reported (Gupta et al., 2009; Laser et al., 2009).

The aim of the present work was to extract maximum sugars with minimum inhibitory compounds from lignocellulosic substrate, *L. camara*, using less stringent acid hydrolysis conditions. We attempted to remove the toxic substances produced during acid hydrolysis employing sequential overliming and activated charcoal adsorption. The delignification of pretreated biomass was carried out by a combinatorial approach using sodium chlorite and sodium sulphite. The pentose and hexose sugar hydrolysates were subsequently fermented separately using *P. stipitis* and *S. cerevisiae*.

2. Methods

2.1. Biomass collection and preparation

L. camara was collected from Aravali Hills, University of Delhi South Campus, New Delhi, India. Dried substrate was comminuted by the combination of chipping and milling to attain a particle size of 1–2 mm using laboratory Knife mill (Metrex Scientific Instrumentation Pvt. Ltd., New Delhi, India). The chopped plant material was washed thoroughly with tap water and dried overnight at 60 °C.

2.2. Biomass composition analysis

The chemical composition of *L. camara* was analysed for holocellulose, Klason lignin, pentosans, ash and moisture content. The raw material was consecutively extracted with alcohol–benzene (1:2, v/v) mixture. The extractive-free *L. camara* dust was processed for chemical analyses following the TAPPI (1992) protocols.

2.3. Optimization of dilute acid pretreatment

The optimization of dilute acid hydrolysis was carried out at different temperatures (100–140 °C), time periods (30 and 45 min) and H_2SO_4 concentration (1–5%, v/v) at 10.0% (w/v) solid content. The hydrolysates recovered were filtered through double-layered muslin cloth, and the remaining biomass was washed properly with tap water to a neutral pH. The hydrolysate was analysed for the amounts of sugar, furans and phenolics released; and the leftover biomass was dried overnight at 60 °C prior to delignification.

2.4. Detoxification of acid hydrolysate

The acid hydrolysate of *L. camara* was mixed with calcium hydroxide to raise the pH of the hydrolysate to 10.0. The whole slurry was stirred for 30 min by an overhead stirrer (Remi Motors Ltd., Mumbai, India). After bringing to room temperature, the hydrolysate was neutralized with concentrated H_2SO_4 and then centrifuged (10,000*g*, 15 min). The overlimed hydrolysate was further detoxified by mixing 1.5% (w/v) activated charcoal with continuous stirring for 30 min at room temperature and the resulting sugar was recovered using vacuum filtration.

2.5. Delignification

The acid-pretreated residue of *L. camara* was treated with different dosages of sodium sulphite (5.0–20.0% w/v) alone and in combination with sodium chlorite (3.0% w/v) at different temperatures (100–140 °C) and time intervals (30 and 45 min). The delignified biomass was then filtered through double-layered muslin cloth and the cellulosic residue was washed thoroughly with tap water until a neutral pH was achieved and dried overnight at 60 °C.

2.6. Enzyme hydrolysis

Commercial cellulase (6.0 FPU/mg) from *Trichoderma reesei* (ATCC 26921) and β-glucosidase (250 U/mL) from *Aspergillus niger* (Novozyme 188) were purchased from Sigma (St. Louis, MO, USA).

The delignified cellulosic residue of *L. camara* was suspended in 0.05 M citrate phosphate buffer (pH 5.0) at 50 °C with a solid content of 5% (w/v) and soaked in a rotary incubator shaker (Innova-40, New Brunswick Scientific, NJ, USA) for 2 h. The soaked suspension was further supplemented with cellulase (3 FPU/mL) and β-glucosidase (Novozyme 188) (9 U/mL). Enzymatic hydrolysis was performed at 50 °C and 150 rpm for 36 h. A dose of 0.005% sodium azide was introduced to avoid any microbial contamination; and 1.0% (v/v) of Tween 80 was added to facilitate the enzymatic action. Samples were withdrawn every 4 h and subsequently analysed for glucose released in the reaction mixture.

2.7. Ethanol fermentation

2.7.1. Microorganisms

P. stipitis NCIM 3498 was obtained from National Collection of Industrial Microorganism (NCIM), National Chemical Laboratory (NCL), Pune, India and was maintained in a medium having (g/L): xylose, 20; yeast extract, 3; peptone, 5; malt extract, 3; agar, 20 at pH 5.0 ± 0.2 and temperature 30 °C (Nigam, 2001). The *S. cerevisiae* strain from our laboratory was maintained in medium containing (g/L): glucose, 30; yeast extract, 3; peptone, 5; agar, 20 at pH 6.0 ± 0.2 and temperature 30 °C (Chen et al., 2007).

The *P. stipitis* inoculum 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. *S. cerevisiae* inoculum was developed by growing the cells at 30 °C for 24 h in a culture medium containing (g/L): glucose, 30.0; yeast extract, 3.0; peptone, 5.0; $(NH_4)_2HPO_4$, 0.25; pH 6.0 ± 0.2 (Chen et al., 2007). Cells were grown to an optical density (OD_{600}) of 0.6.

2.7.2. Fermentation

The fermentation of acid and enzymatic hydrolysates was carried out separately in an *in situ* sterilizable 12 L fermentor (B-Lite Sartorius India Ltd., Bengaluru, India) with a working volume of 10 L. The acid hydrolysate (16.83 g/L sugars) supplemented with (g/L): NH_4Cl, 0.5; KH_2PO_4, 2.0; $MgSO_4{\cdot}7H_2O$, 0.5; yeast extract, 1.5; $CaCl_2{\cdot}2H_2O$, 0.1; $FeCl_3{\cdot}2H_2O$, 0.1; $ZnSO_4{\cdot}7H_2O$, 0.001 (pH 5.5 ± 0.2), was inoculated with 10% (v/v) inoculum of *P. stipitis* (OD_{600} 0.6) and incubated at 30 °C for 36 h under shaking conditions (150 rpm). The cellulosic hydrolysate (34.75 g/L sugars, pH 6.0 ± 0.2) aided with 3 g/L yeast extract and 0.25 g/L $(NH_4)_2HPO_4$ was inoculated with *S. cerevisiae* (10.0% v/v). The pH of the medium was adjusted with 2 N HCl and 2 N NaOH. The silicone-based antifoaming agent (10.0% v/v) was used to control the foaming, whenever required. The dissolved oxygen concentration was monitored continuously throughout the process using a dissolved oxygen probe and a air flow at 0.4 L/min (lpm) was maintained through out the study. Samples were withdrawn at regular intervals of 4 h and centrifuged at 10,000*g* for 15 min at 4 °C. The cell free supernatant was used to determine the ethanol and residual sugar concentration.