Bioresource Technology 100 (2009) 3914–3920

Contents lists available at ScienceDirect

Bioresource Technology

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

Enhanced ethanol production by fermentation of rice straw hydrolysate without detoxification using a newly adapted strain of *Pichia stipitis*

Chiung-Fang Huang, Ting-Hsiang Lin, Gia-Luen Guo *, Wen-Song Hwang

Cellulosic Ethanol Project, Institute of Nuclear Energy Research, Executive Yuan No. 1000, Wunhua Rd., Jiaan Village, Longtan Township, Taoyuan County 32546, Taiwan, ROC

ARTICLE INFO

Article history:
Received 15 October 2008
Received in revised form 19 February 2009
Accepted 20 February 2009
Available online 5 April 2009

Keywords:
Rice straw
Pichia stipitis
Adaptation
NaOH neutralization
Overliming detoxification

ABSTRACT

An enhanced inhibitor-tolerant strain of *Pichia stipitis* was successfully developed through adaptation to acid-treated rice straw hydrolysate. The ethanol production obtained by fermentation of NaOH-neutralized hydrolysate without detoxification using the adapted *P. stipitis* was comparable to fermentation of overliming-detoxified hydrolysate. The ethanol yield using the adapted *P. stipitis* with both types of hydrolysate at pH 5.0 achieved 0.45 $g_p\ g_s^{-1}$, which is equivalent to 87% of the maximum possible ethanol conversion. Furthermore, the newly adapted *P. stipitis* demonstrated significantly enhanced tolerance to sulfate and furfural despite the fact that both inhibitors had not been removed from the hydrolysate by NaOH neutralization. Finally, the ethanol conversion could be maintained at 60% and above when the neutralized hydrolysate contained 3.0% sulfate and 1.3 g L^{-1} furfural.

1. Introduction

The Taiwan government has aggressively promoted an E3 low ethanol-blended fuel since 2007. At the same time, the Institute of Nuclear Energy Research (INER), a governmental organization in Taiwan, has funded the development of cellulosic ethanol production from local lignocellulosic materials. Rice straw, the most abundant agriculture residue produced in Taiwan, has been selected as the most favorable locally available feedstock for ethanol production (Guo et al., 2008).

Prior to ethanol fermentation by a microorganism, the feedstock needs to be processed by saccharification technology in order to release fermentable sugars. To date, dilute sulfuric acid hydrolysis is thought to be one of the promising pretreatment methods and was extensively employed in industry. Except for improving the conversion of cellulose to glucose in following enzymatic hydrolysis, this pretreatment technology was able to produce acceptable xylose yields of 75–90% by conversion of hemicellulose (Eggeman and Elander, 2005; Mosier et al., 2005; Sun and Cheng, 2002). In addition to fermentable sugars, the degradation products include weak acids and furan derivatives, which are generated as part of the pretreatment process. These compounds have been characterized as inhibitors of microbial growth and these seem to affect the fermentation performance. Therefore, the detoxification of the lignocellulosic hydrolysate or the use of an inhibitor-tolerant microorganism is usually required for successful hydrolysate fermentation process (Almeida et al., 2007; Palmqvist and Hähn-Hägerdal, 2000a; Sánchez and Cardona, 2008).

Overliming is a well-established detoxification method that has been applied widely to the acid pretreatment of hydrolysate for ethanol production. This process has been demonstrated to help with the removal of volatile inhibitory compounds such as furfural and hydroxymethyl furfural (HMF) from the hydrolysate (Martinez et al., 2000; Ranatunga et al., 2000). However, overliming is not an effective way of reducing toxicity caused by organic acids like formic acid and acetic acid (Larsson et al., 1999; Ranatunga et al., 2000; Palmqvist and Hähn-Hägerdal, 2000a). Furthermore, overliming treatment usually leads to loss of sugars, which reduces the efficiency of the fermentation; this loss of sugars is mostly caused by their destruction during the stage in the process where the pH is significantly elevated (Martinez et al., 2000; Nilvebrant et al., 2003). A further problem associated with overliming is the need for additional facilities and process systems to remove the gypsum precipitate that is produced as a byproduct; this significantly increases the capital investment needed for the ethanol production plant (Cardona and Sánchez, 2007). Thus, from the economic viewpoint, the development of a robust microorganism that is able to ferment hydrolysate to ethanol without detoxification would be highly important and very useful.

A number of yeast species, including *Pichia stipitis*, *Candida shehatae* and *Pachysolen tannophilus*, have been found to be highly efficient xylose-fermenting strains that can be used in ethanol production (Agbogbo et al., 2006; Jeffries and Jin, 2000; Sánchez

* Corresponding author. Tel.: +886 3 4711400 5039.
E-mail address: glguo@iner.gov.tw (G.-L. Guo).

0960-8524/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biortech.2009.02.064

and Cardona, 2008). Among them, *P. stipitis* exhibits the great potential for industrial application because this species produces a high ethanol yield and has no requirement for added vitamins during the fermentation (Agbogbo et al., 2006; Nigam, 2001a). However, *P. stipitis* is sensitive to organic acids, including acetic acid, which are present in lignocellulosic hydrolysate. These compounds reduce cell growth and diminished ethanol production. As a result, increasing the tolerance of this yeast to such inhibitors would be beneficial to the development of *P. stipitis* as part of cellulosic ethanol production (Almeida et al., 2007; Nigam, 2001b; Palmqvist and Hähn-Hägerdal, 2000b).

The objective of this study was to develop a xylose-fermenting yeast strain of *P. stipitis* that showed enhanced inhibitor tolerance during fermentation of rice straw hydrolysate that had not undergone detoxification. In this report, we describe the development of a hydrolysate-adapted strain of *P. stipitis* obtained by a directed evolution strategy. The performance of the adapted *P. stipitis* during fermentation of overliming-detoxified hydrolysate and NaOH-neutralized rice straw hydrolysate without detoxification was compared in order to understand the potential of this adapted strain as part of an ethanol production process. Specifically, an improvement in the tolerances of the adapted *P. stipitis* to sulfate and furfural were demonstrated. To the best of our knowledge, this is the first report that has comprehensively investigated the fermentation of acid-pretreated rice straw hydrolysate without detoxification using a newly adapted *P. stipitis* strain.

2. Methods

2.1. Microorganism and culture

P. stipitis BCRC21777 was obtained from the Bioresource Collection and Research Centre (BCRC) of Taiwan. The stock culture was grown at 30 °C for 2 days on YPX-agar plate containing 10 g L^{-1} yeast extract, 20 g L^{-1} peptone, 20 g L^{-1} xylose and 20 g L^{-1} agar (Merck, Darmstad, Germany). A colony from the plate was then transferred by loop to a 250 mL Erlenmeyer flask containing 50 ml of YPX growth medium. The inoculated culture was grown at 30 °C with agitation at 100 rpm on an orbital shaker for at least 24 h prior to use. The growth medium consisted of 10 g L^{-1} yeast extract, 20 g L^{-1} peptone, 20 g L^{-1} xylose, and the pH of the medium was 6.6 ± 0.1.

2.2. Strain adaptation to rice straw hydrolysate

The adaptation media were prepared using YP medium (composed of 10 g L^{-1} yeast extract and 20 g L^{-1} peptone), which was mixed with a designed fraction of rice straw hydrolysate, and the sugars levels adjusted by adding synthetic sugars to reach 10 g L^{-1} glucose and 30 g L^{-1} xylose in each medium. The pH of the media was adjusted to 5.0 with 10 N NaOH. No additional nutrient salts or vitamins were added as supplements to the adaptation media.

Strain adaptation was accomplished by sequential transfer of culture samples to adaptation media containing increasing concentrations of neutralized hydrolysate. *P. stipitis* was first grown in the adaptation medium containing 20% hydrolysate with 100 rpm agitation at 30 °C in a 250 mL Erlenmeyer flask. Any surviving microorganisms were then transferred into a fresh adaptation medium with a higher concentration (gradually increasing concentration to 35%, 50%, 60%, 70%, 80%, and 100%) of hydrolysate. The adaptation procedure was performed sequentially until the culture was able to tolerate rice straw hydrolysate without dilution. After this point, the adapted culture was continuously sub-cultured in undiluted neutralized rice straw hydrolysate more than 60 subcultures. Culture growth was monitored at each step by measuring the absorbance at 600 nm (OD_{600}).

2.3. Preparation of rice straw hydrolysate

Rice straw was collected from fields near Longtan, Taoyuan County, Taiwan. Prior to dilute acid hydrolysis, the raw straw material was sliced to a suitable size (between 1 and 2 cm) and dried at 105 °C overnight to ensure a low content of moisture. The rice straw hydrolysate was prepared using a twin-screw conveyer connected to a high-pressure hydrolysis reactor. The sliced rice straw was first introduced into the twin-screw conveyer at 50% solid content soaked with 1–3% (w/w) of dilute sulfuric acid. The straw fed was screwed squeezed at 40 rpm and 145 °C for 20 min, then the mixtures was transferred to the high-pressure hydrolysis reactor. Low-pressure steam was employed immediately to increase the reaction temperature to 130 °C and the reaction was continued for 15 min, which resulted in the final solid content of about 30%. The treated straw material and hydrolysate were drained out from the processor outlet and quickly separated by filtration. The filtrated rice straw hydrolysate was reserved and used for the fermentations that form part of this study.

2.4. Fermentation of the rice straw hydrolysate

The fermentation studies were performed using both overliming-detoxified hydrolysate and the neutralized hydrolysate without detoxification. For the preparation of the overliming-detoxified hydrolysate, the hydrolysate was first heated to 50 °C and held at this temperature for 15 min; this was followed by the addition 18 g L^{-1} of calcium hydroxide (lime), which raised the pH of the hydrolysate to 10.0. Agitation was then carried out for 30 min. The calcium sulfate ($CaSO_4$) sludge and the liquid were next separated by filtration and finally the pH of the filtered overliming-detoxified hydrolysate was adjusted with sulfuric acid (H_2SO_4) to 5.0. In contrast, the neutralized hydrolysate was prepared by directly adjusting the pH of the filtered hydrolysate to 5.0 with 10 N NaOH. Both the overliming-detoxified and NaOH-neutralized hydrolysates were autoclave-sterilized and then used for the various fermentation studies. Culture inoculate were grown in 2% YPX for at least 24 h prior to harvesting by centrifugation. Fermentations were carried out in 250 mL Erlenmeyer flasks containing 60 mL of fermentation medium and were performed at 30 °C and 100 rpm using a rotary shaker. The initial cell concentration was set at 1.5 g L^{-1}.

In addition to the above media, inhibitor-supplemented fermentation media were prepared by adding designated amounts of sulfuric acid or furfural to NaOH-neutralized hydrolysate. The fermentation parameters for the inhibitor-supplemented hydrolysate were the same as above except the initial cell concentration was set at 0.7 g L^{-1}. Moreover, no nutrient salts or vitamins were added in all fermentation media. All fermentation experiments were performed in triplicate, and samples were taken periodically for HPLC analysis.

2.5. Analytical methods

Each sample was filtered through a 0.22 μm filter and diluted appropriately with deionized-water. Quantitative analysis for glucose, xylose, arabinose, xylitol, acetic acid, ethanol, hydroxymethyl furfural/HMF and furfural was performed on an Agilent 1100 HPLC system (Agilent, Palo Alto, CA, USA) at 45 °C and equipped with a refractive index detector. The separation was achieved using a Coregel-87H3 column (Transgenomics, Co) maintained at 65 °C with 4 mM H_2SO_4 as eluent and at a flow rate of 0.8 mL min^{-1}.