Bioresource Technology 121 (2012) 369–378

Contents lists available at SciVerse ScienceDirect

Bioresource Technology

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

Effects of lignin-derived phenolic compounds on xylitol production and key enzyme activities by a xylose utilizing yeast *Candida athensensis* SB18

Jinming Zhang [a,b], Anli Geng [b,*], Chuanyi Yao [a], Yinghua Lu [a,c], Qingbiao Li [a,c]

[a] Department of Chemical and Biochemical Engineering, College of Chemistry and Chemical Engineering, Xiamen University, Xiamen 361005, PR China
[b] School of Life Sciences and Chemical Technology, Ngee Ann Polytechnic, Singapore
[c] The Key Lab for Chemical Biology of Fujian Province, Xiamen 361005, PR China

HIGHLIGHTS

- Xylitol producer *Candida athensensis* SB18 was tested for phenolic compound inhibition.
- The inhibition follows phenol > syringaldehyde > 4-hydroxylbenzaldehyde > vanillin.
- Inhibition was insignificant when the total inhibitor content was below 1.0 g/L.
- The inhibitors affected more xylose reductase than xylitol dehydrogenase activity.
- The inhibitory effects strongly correlated to their in vivo assimilation.

ARTICLE INFO

Article history:
Received 29 March 2012
Received in revised form 3 July 2012
Accepted 5 July 2012
Available online 14 July 2012

Keywords:
Candida athensensis SB18
Phenolic compound
Inhibitors
Xylose reductase
Xylitol dehydrogenase

ABSTRACT

Candida athensensis SB18 is potential xylitol producing yeast isolated in Singapore. It has excellent xylose tolerance and is able to produce xylitol in high titer and yield. However, by-products, such as phenolic compounds, derived in lignocellulosic biomass hydrolysate might negatively influence the performance of this strain for xylitol production. In this work, four potential phenolic inhibitors, such as vanillin, syringaldehyde, 4-hydroxybenzaldehyde and phenol, were evaluated for their inhibitory effects on xylitol production by *C. athensensis* SB18. Phenol was shown to be the most toxic molecule on this microorganism followed by syringaldehyde. Vanillin and 4-hydroxylbenzaldehyde was less toxic than phenol and syringaldehyde, with vanillin being the least toxic. Inhibition was insignificant when the total content of inhibitors was below 1.0 g/L. The presence of phenolic compounds affected the activity of xylose reductase, however not on that of xylitol dehydrogenase. *C. athensensis* SB18 is therefore a potential xylitol producer from hemicellulosic hydrolysate due to its assimilation of such phenolic inhibitors.

1. Introduction

As a natural five carbon sugar alcohol with sweetness, xylitol has been increasingly used in food and pharmaceutical industries due to several advantages. Recently, the demand for xylitol in food industries as an alternative sweetener has created a strong market for the development of low-cost xylitol production processes. With the increased interests in alternative energy and biochemical sources, lignocellulosic materials are becoming attractive as a potential low-cost feedstock for the production of biofuels and value-added chemicals such as bioethanol and xylitol. Lignocellulosic materials contain polysaccharides such as celluloses and hemicelluloses. The hydrolysate of the hemicellulosic fraction containing mainly D-xylose can be used as the substrate for xylitol production by chemical or biotechnological means (Nigam and Singh, 1995). Currently, the most promising path for xylitol production from xylose rich hemicellulosic feedstock is microbial fermentation (Converti et al., 2000; Carvalho et al., 2003; Rodrigues et al., 2006). Hemicellulosic hydrolysate can be obtained by chemical pretreatment of the lignocellulosic biomass. Organosolv and dilute acid pretreatment methods are among the most commonly used and effective pretreatment means (Geng et al., 2003; Villarreal et al., 2006). Such methods usually involve the operation under high temperature and pressure. Under such conditions, several by-products are derived from sugars and lignin; they are liberated in the hemicellulosic hydrolysate. These compounds include three major groups: (1) furan derivatives (furfural and 5-hydroxymethyl

* Corresponding author. Address: School of Life Sciences and Chemical Technology, Ngee Ann Polytechnic, 535 Clementi Road, Singapore 599489, Singapore. Tel.: +(65) 64608617; fax: +(65) 64679109.
E-mail address: gan2@np.edu.sg (A. Geng).

0960-8524/$ - see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.biortech.2012.07.020

furfural (HMF)); (2) weak acids (mainly acetic acid, formic acid and levulinic acid); (3) phenolic compounds such as vanillin, syringaldehyde, 4-hydroxybenzaldehyde, and phenol (Buchert et al., 1990; Almeida et al., 2007). As reported, furan derivatives are released from the further degradation of pentose (furfural) and hexose (HMF), while weak acids are generated when furfural and HMF are further degraded (Ulbricht et al., 1984), and phenolic compounds on the other hand are produced from the partial breakdown of lignin (Sears et al., 1971).

The above by-products derived from sugars and lignin negatively affect the fermentation efficiency due to their toxic influence on the fermentative microorganisms (Mussatto and Roberto, 2004). The type and concentration of the compounds existing in the hydrolysates depend on the type of raw materials and the pretreatment conditions. Their inhibitory effects vary with their concentration and the microorganisms (Martin and Jönsson, 2003). In order to improve the fermentative efficiency and to enhance the metabolic activity of the microorganisms, a greater understanding of the inhibitory mechanisms of the individual toxic compounds, their interactive effects, and the in vivo degradation possibility of these compounds is required. Among these toxic compounds liberated during the pretreatment process, weak acids were proven to inhibit cell growth of microorganisms due to the inflow of undissociated acid into the cytosol (Stouthamer, 1979; Verduyn et al., 1992; Russell, 1992). Furan derivatives were shown to reduce the specific growth rate and the cell biomass yield, and they can be converted to their corresponding alcohols (Palmqvist et al., 1999; Palmqvist and Hägerdal, 2000). While phenolic compounds have been suggested to exert a considerable inhibition in the fermentation process due to its ability to affect cell membranes to serve as selective barriers and enzyme matrices (Heipieper et al., 1994), their inhibition mechanisms on microorganisms have not yet been completely elucidated, largely due to the heterogeneity of the group and the lack of accurate qualitative analyses. 4-hydroybenzoic acid, vanillin, and catechol were identified as the major constituents in the willow hemicellulose hydrolysate by Jönsson et al. (1998) and their toxicity can be reduced by laccase treatment. Vanillin constitutes a major fraction of the phenolic monomers in the hemicellulosic hydrolysate of softwood, such as spruce, pine and willow (Palmqvist and Hägerdal, 2000) and its presence (1 g/L) decreased the ethanol yield by 25% (Ando et al., 1986). A variety of aromatic monomeric compounds such as vanillin, syringaldehyde, 4-hydroxybenzaldehyde and 5-hydroxylmethylfurfural were identified to be present in the hemicellulosic hydrolysate of birchwood (Buchert et al., 1990) and the total concentration of such phenolics could reach about 2 g/L (Clark and Mackie, 1984). The presence of such phenolic compounds was shown to inhibit yeast growth and therefore reduce xylitol production to different degrees depending on their concentration (Kelly et al., 2008; Cortez and Roberto, 2010b).

Candida athensensis SB18 is potential xylose-producing yeast isolated in Singapore with excellent xylose tolerance and xylitol production capabilities. In order to investigate the potential of this strain in converting of hemicellulosic sugars to xylitol, the present work evaluates the inhibitory effects of the most often detected phenolic by-products, such as vanillin, syringaldehyde, 4-hydroxybenzaldehyde and phenol on the fermentative behavior and enzyme activities of the new isolate *C. athensensis* SB18. The effects of both individual and combination of these inhibitors on cell growth, xylitol production and the key enzymes activities are studied. In addition, phenolic compound degradation is performed in order to better understand of the inhibitory mechanism of such phenolic compounds. Such knowledge will be useful for the development and optimization of a lignocellulosic biomass pretreatment method and for the application of *C. athensensis* SB18 in industrial xylitol production.

2. Methods

2.1. Microorganism and inoculum cultivation

The xylitol producing yeast *C. athensensis* SB18 was isolated in Singapore soil samples (Zhang et al., 2012) and was maintained on YPX-agar plates containing (g/L): xylose (Merck, Germany), 20; yeast extract (Merck, Germany), 10; peptone (Difco, USA); and agar (Merck, Germany), 20. Inoculum was prepared by transferring the cells from the 24 h YPX agar plates to 250-mL shaking flasks containing 100 mL seed culture medium consisting of (g/L): xylose, 50; yeast extract, 10; and peptone, 20. Flasks were incubated at 150 rpm and 30 °C for 24 h. Cells were harvested by centrifugation at 8000*g* and 4 °C for 4 min. They were then washed twice using sterile water and stored at 4 °C until use as the inoculum.

2.2. Medium and fermentation condition

Experiments were carried out in 100-mL Erlenmeyer's flasks containing 40 mL of the fermentation medium composed of (g/L): xylose, 50; yeast nitrogen base (YNB), 6.76; yeast extract, 1.0; and urea, 2.0. All the nutrients were prepared separately and were autoclaved at 121 °C for 15 min. YNB and urea were sterilized through 0.2 μm filter. Flasks were inoculated with an inoculum size of 1.5 g/L (unless otherwise stated) and incubated at 30 °C for 4–7 days. Fermentation experiments were conducted in the presence of different inhibitors at varied concentrations. Samples were withdrawn periodically to determine the cell density at 600 nm (OD_{600}) and the concentration of residual substrates and products. All experiments were conducted in duplicate.

2.3. Inhibitor cocktail

The stock solution of all the inhibitors including syringaldehyde (Syr), 4-hydroxybenzaldehyde (Hba), vanillin (Van) and phenol (Phe), was prepared by dissolving the desired mass of each phenolic compound in the sterile fermentation medium. They were then re-sterilized through 0.2 μm filter. The initial inhibitor concentration in the stock solution was (g/L): Syr, 2.0; Van, 2.0; Hba, 1.0; and Phe, 1.0, individually. The inhibitor cocktail were freshly prepared by mixing the individual inhibitor stock solution to the desired concentration in the fermentation medium.

2.4. Crude enzyme extraction

Samples were withdrawn periodically during fermentation process and cells were harvested by centrifugation at 8000*g* and 4 °C for 4 min. Cell pellets were washed twice with cold sterile distilled water and re-suspended with 0.25 M potassium phosphate buffer (pH 7.0) supplemented with 0.1 M 2-mercaptoethanol. The final cell suspension (about 3 g/L) was mechanically disrupted in 2 mL eppendorf tubes under vortex mixing using glass beads with a particle size of 0.5 mm in a volumetric ratio of 1:1 (1 mL of cell suspension to 1 mL of glass beads). Cell disruption was conducted for 5 min with 20 s of disruption followed by 30 s intervals on ice (Gurpilhares et al., 2006). The resulted solution was then centrifuged at 8000*g* and 4 °C for 6 min and the supernatants were assayed for the activities of xylose reductase (XR) and xylitol dehydrogenase (XDH).

2.5. Enzyme activity assay

Xylose reductase (XR) and xylitol dehydrogenase (XDH) activities were determined by measuring the absorbance at 340 nm